Immunoisolation of a Golgi subcompartment from *Saccharomyces cerevisiae*

Thesis for the degree of Doctor of Philosophy

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I would like to dedicate this thesis to my
mother, Valerie Owen, whose endless supply of love, support,
poems and vitamin pills never ceases to amaze me

Declaration

The work presented in this manuscript was carried out under the supervision of Dr. Alan Boyd in the Department of Biochemistry, University of Edinburgh between October 1989 and November 1992.

The experimental work presented in this thesis, unless stated otherwise, is my own; and this manuscript has been composed by myself.

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Abstract

Although no structure morphologically similar to the well-characterised Golgi complex of mammalian cells can be seen in *Saccharomyces cerevisiae* (yeast), there is much genetic and biochemical evidence to suggest the existence of a yeast organelle functionally equivalent to the mammalian Golgi. The *trans* Golgi network (TGN) of mammalian cells is characterised, at least in part, by the presence of serine proteases that process prohormone molecules by catalysing cleavage of polypeptide chains at pairs of basic residues. Analogous proprotein processing in yeast is achieved by the action of the Kex2 protease. Kex2p is the best-characterised resident protein of the yeast Golgi to date, and is believed to reside in a compartment of the organelle that is equivalent to the mammalian TGN.

In order to further characterise the yeast Golgi, the aim of this project was to prepare vesicles derived specifically from the Kex2p-containing compartment of the yeast Golgi. This aim was pursued in two ways. Firstly, a hybrid protein, consisting of IgG-binding domains of the *S. aureus* coat protein (protein A) fused to the extreme, cytoplasmically-disposed, C-terminus of Kex2p was constructed. It was intended that IgG-Sepharose would be used to isolate membrane vesicles containing the hybrid protein from yeast cell lysates, and that these vesicles would originate from the Kex2-containing compartment. Unfortunately, this approach was unsuccessful since preliminary results suggest that the protein is mislocalised to the vacuole.

The second approach that was employed involved the generation of a polyclonal antibody preparation that specifically recognises the cytoplasmically-disposed C-terminal region of Kex2p. This was achieved using bacterially-produced hybrid proteins (containing the C-terminal 100 residues of Kex2p) to immunise rabbits, and to affinity-purify Kex2p specific antibodies from immune serum. Affinity-purified antibodies were bound to fixed *S. aureus* cells by taking advantage of the high affinity of protein A for the Fc portion of IgG molecules. The resulting immunoadsorbent (ImAd) can be used to recover 80% of Kex2 protease activity from a yeast cell lysate. Kex2p recovered using the procedure established during the course of this project is contained within intact membrane vesicles as demonstrated by its resistance to externally added protease and the ability of the ImAd to recover a soluble cargo protein. As well as containing Kex2p the material bound by the ImAd is enriched for dipeptidyl aminopeptidase A and Kex1p, both of which reside in the same compartment as Kex2p in the cell, but does not contain activities of enzymes associated with the endoplasmic reticulum (NADPH cyt c reductase) or the vacuole (dipeptidyl aminopeptidase B).

The material that has been immunoisolated using the Kex2p antibody will be of use to provide insights into the structure of the yeast Golgi, as well as being central to the development of protein transport assays to study the function(s) of this organelle.

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Abbreviations used in this thesis

A_{405}	absorbance at 405nm
ADP	adenosine 5'-diphosphate
AMC	7-amino-4-methylcoumarin
ATP	adenosine 5'-triphosphate
βgal	β-galactosidase
bp	base pair
BSA	bovine serum albumin
b-QRRMCA	t-butoxycarbonyl-Gln-Arg-Arg 4-methylcoumarin-7-amide
CHO	chinese hamster ovary
Ci	Curie
COP	coat protein
CPY	carboxypeptidase Y
cyt *c*	cytochrome *c*
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNP	di-nitro phenol
Dol	dolichol
DPAP	dipeptidyl aminopeptidase
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	diaminoethanetetra-acetic acid
EGTA	1,2-di(2-aminoethoxy)ethane-N,N,N',N'-tetra acetic acid
endo H	endoglycosidase H
ER	endoplasmic reticulum
g	relative centrifugal force
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate

Abbreviations (continued)

GTPγS	guanosine 5'-O-(3-thiotriphosphate)
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	horseradish peroxidase
Ig	immunoglobulin
ImAd	immunoadsorbent
IPTG	isopropylthiogalactoside
K	thousand
D	dalton
kb	kilobase
MDCK	Madin Darby canine kidney
mg	milligram
min	minute
ml	millilitre
MSS	medium speed supernatant
MSP	medium speed pellet
MWt	molecular weight
μg	microgram
μl	microlitre
NEM	*N*-ethylmaleimide
nm	nanometers
NSF	NEM sensitive factor
^{o}C	degrees centigrade
OD_{600}	optical density at 600nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

Abbreviations (continued)

PC	phosphatidylcholine
PI	phosphatidylinositol
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
S. aureus	*Staphylococcus aureus*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SDS	sodium dodecyl sulphate
Spa	Staphylococcal protein A
SRP	signal recognition particle
SV	secretory vesicles
TBS	Tris buffered saline
TCA	trichloroacetic acid
TGN	*trans* Golgi network
Tris	2-amino-2-(hydroxymethyl) propane-1,3,-dio(tris)
TST	Tris-saline-Tween-20
Tween-20	Polyoxyethylene sorbitan monolaurate
VSV	vesicular stomatitus virus
WCE	whole cell extract

Abbreviations for aminoacids

Amino Acid	Abbreviations	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamate	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter 1

Introduction

Section 1. The secretory pathway

1.1. Introduction and overview

Eukaryotic cells differ in many ways from their prokaryotic counterparts. One of their most striking features in this respect is their intracellular compartmentalisation (Alberts *et al.*, 1989). The existence of organelles, which are created by enclosing various groups of molecules within membranes of a defined lipid and protein compostion, allows processes which would otherwise interfere with each other to occur within the same cell. For example, proteolytic enzymes are contained within the lysosome of mammalian cells (Alberts *et al.*, 1989) or the vacuole of yeast cells (Wiemken *et al.*, 1979) away from other cellular components which they would otherwise degrade. In order for cells to achieve and maintain this compartmentalisation it is vital that new proteins reach their desired locations so that the function for which they have been produced occurs in the correct place and not in any other. In other words the cell must be able to target proteins accurately to their rightful cellular locations.

Proteins destined for many organellar locations, as well as those to be secreted from the cell travel through the secretory pathway (for review see Kelly, 1985). Entry into this pathway is achieved by proteins being translocated across, or inserted into, the ER membrane. From the ER, proteins travel to and subsequently enter the Golgi apparatus. This transfer occurs by means of vesicular traffic (Palade, 1975): membrane buds from the ER to form vesicles entrapping soluble proteins present in the ER lumen, then delivery of cargo proteins is achieved as these vesicles fuse with the *cis* cisternae of the Golgi apparatus, consequently becoming incorporated into its membrane and delivering any contents inside. Transport through the different cisternae of the Golgi complex is also mediated by vesicular traffic and proteins leave this organelle when they are packaged into secretory vesicles which bud from the *trans* face of the Golgi. These secretory vesicles can then fuse with the plasma membrane in order to deliver proteins to the cell surface, or to secrete soluble proteins from the cell;

alternatively they may fuse with the lysosomal (or vacuolar) membrane to achieve delivery of proteins to this organelle. In mammalian cells the fusion of post-Golgi secretory vesicles with the plasma membrane occurs both constitutively and in a regulated manner (for review see Burgess and Kelly, 1987). Regulated secretory cells, such as neurones and cells of the endo- and exocrine glands, are specialised to release, over a short period of time, large amounts of protein at a much higher rate than they are synthesised. Cells of the endocrine system concentrate secretory proteins by as much as 200-fold during their passage from the *trans* Golgi to secretory vesicles (Salpeter and Farquhar, 1981). Such proteins are stored in specialised vesicles close to the plasma membrane until their exocytosis is required. Fusion of these vesicles with the plasma membrane, and therefore the release of their contents, is triggered by a specific external stimulus in response to a change in the physiological state of the organism and is mediated through changes in the concentration of an intracellular messenger such as calcium (Burgoyne, 1990). In contrast, the constitutive secretory pathway does not concentrate newly synthesised molecules; there is no post-Golgi storage pool and secretory vesicles move directly from the Golgi to the plasma membrane where fusion occurs. The constitutive pathway is thought of as the general route in all cells for the transport of molecules that are constantly required at the cell's exterior such as growth factors, extracellular components and enzymes. There is direct evidence that both regulated and constitutive secretion occur within the same cell. Proteins that are secreted from a cell constitutively are found in transport vesicles distinct from those containing protein that is secreted only in response to an external stimulus (Gumbiner and Kelly, 1982).

Current views of protein traffic favour the theory that proteins entering the ER travel through the secretory pathway as described above, with no specific sequence being required for a protein to be moved from the ER to the Golgi, or for entry into post Golgi secretory vesicles and that it is proteins required at other cellular locations such as the lysosome or vacuole that carry sequences containing the information necessary to direct them away from this default pathway (Kornfeld and Mellman, 1989; Pearse and

Robinson, 1990). Similarly, it is believed that proteins resident to the constituent compartments of the pathway carry information which prevents them from being permanently removed from their rightful location by the bulk-flow of membrane traffic. Our understanding of such information remains limited, soluble proteins resident to the ER of mammalian cells contain the C-terminal tetrapeptide -KDEL and those resident to the ER of yeast cells -HDEL (Pelham, 1989). It is believed that these proteins are not retained, as such, in the ER, but rather are recycled from a post-ER compartment by virtue of their C-terminal signal interacting with a receptor. The 'bulk-flow' view of the secretory pathway described above gained support from experiments that followed the movement of small peptides through the secretory pathway of mammalian cells to show that fast and efficient export from the cell does not require a specific signal in the molecule being secreted (Wieland *et al.*, 1987). Small peptides in this system were found to be secreted from the cell with the same kinetics as a number of secretory proteins (Helms *et al.*, 1990). However, the transport route of such small peptides from the ER seems to be dependent on the biological system in which they are used, since similar experiments in *Xenopus* oocytes and yeast show that the peptides fail to be secreted and are transported into the cytoplasm of yeast and the lysosome of the oocytes (Geetha-Habib *et al.*, 1990: Romisch and Schekman, 1992). Recent work in yeast questions the idea of the default pathway as described so far, suggesting that the default pathway for integral membrane proteins in this organism may be delivery to the vacuole. Evidence for this comes from experiments which show that none of the three domains of a protein resident in the vacuole carry information to direct the protein there, and also that the removal of information that directs and keeps a resident Golgi protein in its compartment results in its localisation to the vacuole (Roberts *et al.*, 1992). Such recent findings demonstrate that our understanding in this area is far from complete.

As mentioned above, proteins are carried through the secretory pathway by vesicles which bud off from one membrane bound compartment (the donor) and subsequently fuse with another (the acceptor). Movement of proteins between the organelles of the secretory pathway requires accuracy of fusion of these vesicles with their acceptor

compartments and little is known about how this is achieved within the cell. Assays that reconstitute certain events *in vitro* have proved to be useful as tools to aid the analysis of complicated biological processes, and it is hoped that the reconstitution of stages of the secretory pathway will aid the biochemical dissection of the pathway and lead to the identification and subsequent characterisation of components involved in it.

1.2. Investigation of the yeast secretory pathway

Saccharomyces cerevisiae (yeast) is attractive as an experimental organism in which to study protein transport, a process which has been shown to occur analogously in yeast and higher eukaryotes (Wilson *et al.*, 1989: Rothman and Orci, 1992). The ease with which yeast can be manipulated genetically means that it can be used to examine the pathway not only biochemically (by the development of assays which reconstitute protein transport events *in vitro*), but also genetically, an approach that cannot be considered when working with a mammalian system.

Various stages of protein transport in yeast have been identified and defined by the characterisation of mutant cells which are defective in protein transport at 37°C. Characterisation of such mutants by Novick and co-workers led to the identification of twenty three genes whose functions are required for the secretion of proteins (Novick *et al.*, 1980). At the restrictive temperature of 37°C, cells carrying temperature sensitive mutations in any one of these genes (*sec* mutants) accumulate intracellular pools of secretory proteins not found in the same cells at 25°C. These *SEC* genes were grouped, according to morphological studies, in relation to which organelle proliferated in cells in which their function had been altered. The largest of these groups has ten members, and a mutation in any of these genes causes cells to accumulate membrane vesicles 80-100nm in diameter which were proposed to represent secretory vesicles that would ordinarily fuse with the plasma membrane. A second set of *sec* mutants has nine members all of which accumulate an extended ER network as compared to that found in wild type cells at 37°C. A subset of this second group accumulates 40nm vesicles as

well, and these are known as class II mutants, whereas class I mutants accumulate the ER structure alone (Kaiser and Schekman, 1990). Two of the remaining four *sec* mutants identified were found to accumulate a membraneous stack which has been described as 'an exaggerated Golgi-like structure'. The last two *sec* mutants have not been placed into the above groups since one (*sec19*) appears to accumulate all three types of organelle, and another (*sec11*) did not appear to accumulate any.

The order of events in the yeast secretory pathway was elucidated by the characterisation of strains carrying two different *sec* mutations (Novick *et al.*, 1981). The morphology of the exaggerated organelles accumulated in such cells allowed the order in which the *SEC* gene products are required to be assessed. Examination of precursor forms of accumulated invertase (a protein that is ordinarily secreted from yeast cells) in *sec* mutants allowed the order of processing events performed on the protein to be determined. These studies led to the following model (summarised in Figure 1-1): Secretory proteins enter the ER where they are modified by the addition of sugar chains. Nine or more *SEC* gene products act to transfer secretory proteins to a second membrane compartment where the glycans they received in the ER are modified. The products of at least two genes are then required for proteins to move from this compartment into secretory vesicles. Following this, a further nine or more gene products are involved in the delivery of the secretory vesicles to their final destination.

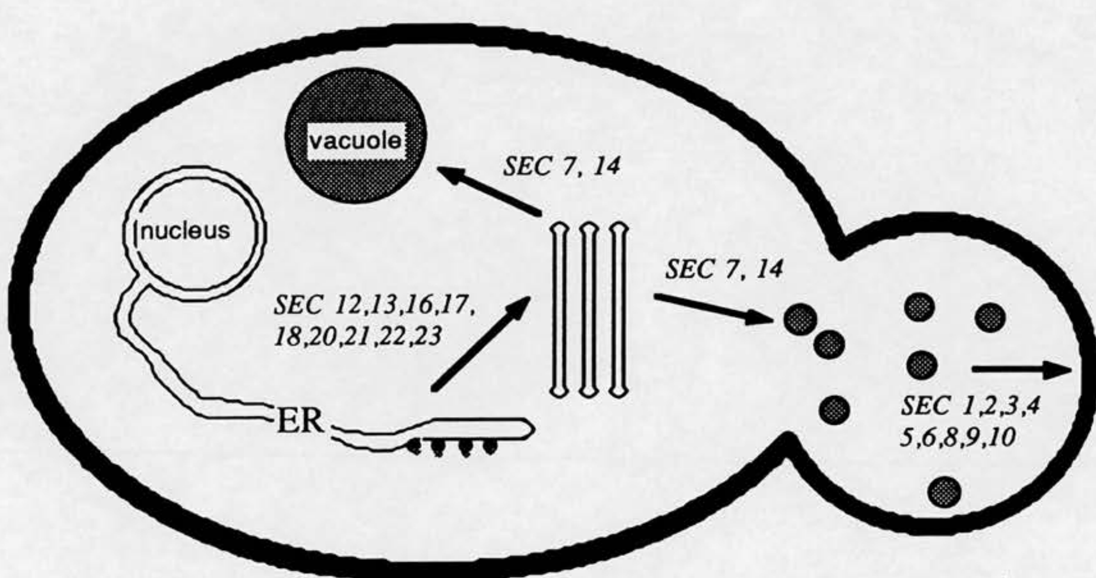
Since the identification of the initial twenty three *SEC* genes, other genes involved in protein transport in yeast have been identified. These include genes whose products are involved in the translocation of proteins across the ER membrane (e.g. *SEC61*: Rothblatt *et al.*, 1989) as well as other genes, mutations in which block the secretory pathway at the stage(s) in which their products are involved (e.g. *BET1* : Newman and Ferro-Novick, 1987) and genes, mutations in which cause the cell to mislocalise certain proteins (e.g. *vps* mutants (Robinson *et al.*, 1988: Rothman *et al.*, 1989) secrete vacuolar proteins). Genes encoding small GTP-binding proteins that are

involved in the yeast secretory pathway, such as *YPT1* (Segev *et al.*, 1988) have also been identified, as have suppressor genes that restore growth to cells bearing mutations in such genes (e.g. *SLY2* and *SLY12*, Dascher *et al.*, 1991: Ossig *et al.*, 1991).

Figure 1-1

The yeast secretory pathway

Proteins that are secreted by yeast follow a pathway similar to that described in mammalian systems. Proteins destined for secretion enter the pathway by translocation across the membrane of the ER after which they are carried through various membrane bound compartments; all transport is mediated by vesicular traffic. Temperature sensitive mutations that block this pathway at precise locations have been isolated. These blocks are reflected in an accumulation of an organelle of the secretory pathway in cells carrying the mutations at 37°C. This figure indicates the position at which the wild type gene products of the twenty three *SEC* genes identified by Novick and his co-workers are thought to function (Novick *et al.*, 1980: Novick *et al.*, 1981).



Section 2. Protein transport assays

2.1. Introduction

Although the use of genetics has yielded much information about the secretory pathway in yeast, it is important that this information is combined with biochemical methods of studying protein transport, since many components involved may not be represented among the products of the known *SEC*, *BET* or *VPS* genes. This is likely to be the case since molecular analyses of yeast chromosome I has revealed that the majority of essential genes are not identified in studies that rely on temperature sensitive mutations (Diehl and Pringle, 1991: Harris and Pringle, 1991). The use of protein transport assays which reconstitute various stages of the secretory pathway *in vitro*, combined with the information gleaned from yeast genetics may aid in the identification of such components as well as providing an insight into their function *in vivo*.

Central to the development of a transport assay is the ability to follow the transport step being investigated. One way in which this can be done is by monitoring a biochemical change known to occur in a protein as it is moved from one compartment to the next. Such changes occur as proteins travel through the secretory pathway, being carried out by specific enzymes that reside in different compartments of the pathway.

2.2. Proteins are modified as they travel through the secretory pathway

Proteins are acted upon by a number of different enzymes as they travel through the secretory pathway and this results in covalent modification of the nascent polypeptide chain. It is these modifications that lead to the differences that exist between the primary translation product of a gene and a mature protein.

Translocation of proteins into or across the ER membrane (for review see Nunnari and Walter, 1992) usually occurs cotranslationally although there are some examples of

proteins crossing the membrane posttranslationally (Schlenstedt *et al.*, 1990), a process more commonly observed in yeast than in mammalian cells (Hansen *et al.*, 1986: Rothblatt and Meyer, 1986). Genes encoding proteins destined to enter the secretory pathway encode a signal sequence which usually resides at the extreme N-terminus of the nascent polypeptide (Blobel and Dobberstein, 1975). This sequence generally includes 1-3 positively charged amino acids followed by a run of 14-20 uncharged and predominantly hydrophobic amino acids and is often ended with a glycine or alanine residue (von Heijne, 1985). When such a signal sequence emerges from a ribosome in mammalian cells it is bound by a cytoplasmic ribonucleoprotein complex, signal recognition particle (SRP) (Walter and Blobel, 1981: Krieg *et al.*, 1986), which arrests any further elongation of the polypeptide chain. SRP, as part of the ribosome-SRP-nascent polypeptide chain complex, interacts with its receptor in the ER membrane (of which docking protein is the largest subunit) and thus directs the complex to the membrane (Meyer *et al.,* 1982: Gilmore *et al.,* 1982). Following this, both SRP and docking protein are released from the ribosome in a reaction requiring GTP, and the elongation of the polypeptide chain continues with its translocation across the membrane having been initiated (Gilmore and Blobel, 1983: Wiedmann *et al.*, 1987). Although yeast homologues of SRP and docking protein have been identified (Amaya *et al.*, 1990: Nunnari and Walter, 1992) the role of translational arrest in yeast is less clearly understood than it is in mammalian cells. This is possibly due to the fact that there may be more than one translocation route into the ER of yeast cells (Hann and Walter, 1991). This would also provide an explanation for the observation that mutations in the *SEC65* gene (whose product shows homology to one of the smaller subunits of SRP) are not lethal (Stirling *et al.*, 1992: Stirling and Hewitt, 1992: Hann *et al.*, 1992), perhaps because proteins can still enter the ER, and therefore the secretory pathway, by another route which usually runs in parallel to the SRP system. In addition to a signal sequence, membrane proteins possess at least one further hydrophobic region to anchor them in the membrane, but if no such region is present (as in soluble proteins) the entire polypeptide chain will pass through the membrane and into the lumen of the ER (Rapoport and Weidmann, 1985).

The actual mechanism by which a polypeptide chain crosses the phospholipid bilayer of the ER is poorly understood, but recent studies in both yeast and mammalian cells have identified several proteins believed to participate in this process. A channel, whose opening can be detected by the passage of glutamate ions has been identified in the ER membrane of mammalian cells (Simon and Blobel, 1991). The opening of this channel depends on ribosome binding to the ER membrane, and can be blocked by the presence of nascent polypeptide chains. These findings support popular theories which suggest the existence of a proteinaceous tunnel to provide a hydrophilic environment allowing the passage of polypeptide chains through the lipid bilayer. The incorporation of photoreactive probes into nascent polypeptide chains has facilitated the identification and purification of proteins with which polypeptides come into contact as they traverse the membrane (Gorlich *et al.,* 1992: and reviewed in Nunnari and Walter, 1992). Similar work in yeast has shown that nascent chains come into close proximity with the products of two genes (*SEC61* and *SEC62*) that were identified by virtue of mutations that disrupt translocation of proteins into the ER (Musch *et al.*, 1992: Sanders *et al.*, 1992). It is hoped that such approaches will eventually lead to the identification and purification of all factors involved in the translocation of polypeptide chains across the ER membrane so that this process can be fully understood.

As a protein enters the ER the signal sequence is usually removed by signal peptidase (Baker *et al.*, 1986; Evans *et al.,* 1986), the yeast homologue of which is encoded by *SEC11* (Bohni *et al* 1988: YaDeau *et al.*, 1991). Proteins undergo other modifications in the lumen of the ER (Hurtley and Helenius, 1989) including the formation of disulphide bonds between cysteine residues and the addition of oligosaccharide chains to selected asparagine residues (for review see Kornfeld and Kornfeld, 1985 and also Tanner and Lehle, 1987). The latter is the initiating step in the formation of the N-linked glycosylation found in mature glycoproteins. A core oligosaccharide chain built up from three glucose, nine mannose and two N-acetyl glucosamine molecules

($Man_9Glc_3GlcNAc_2$) is transferred from the lipid donor dolichol to the amide nitrogen of an asparagine residue present in a polypeptide chain within the sequence Asn-X-Ser/Thr (where X can be any residue except proline). Once attached to the polypeptide chain the $Man_9Glc_3GlcNAc_2$ moiety is processed by other enzymes located in the lumen of the ER.

In yeast cells the removal of the glucose residues and of a single α-1,2-Man residue (by glucosidases and a mannosidase respectively) results in the formation of $Man_8GlcNAc_2$ which is subsequently extended by the addition of up to six mannose residues in a series of reactions catalysed by a mannosyl transferase (for a review of protein glycosylation in yeast see Kukuruzinska *et al.*, 1987). Similar processing occurs in the ER of mammalian cells with glucose and α-1,2-Man residues being removed from the core structure to form the high mannose type glycans found in mammalian glycoproteins.

As well as Asn residues found in the consensus sequence mentioned above, certain Ser and Thr residues found in proteins in the ER of yeast cells are targets for the attachment of oligosaccharides (Haselbeck and Tanner, 1983). These receive a single mannosyl residue from a dolichol carrier and this marks the initiation of the O-linked glycosylation found in yeast glycoproteins. (N.B. This process does not occur in the ER of mammalian cells, O-linked glycosylation is initiated in the Golgi apparatus of such cells, Niemann *et al.*, 1982: Johnson and Spear, 1983). No consensus target sequence for this process has been identified for the attachment of O-linked sugars but regions rich in Ser and Thr residues do seem to be particularly susceptible to this modification.

Polypeptide chains fold into their native conformations and, if necessary assemble into competent strucures by association with other polypeptide chains within the ER lumen. Chains which fail to do so are prevented from leaving the organelle by resident ER proteins (e.g. BiP) and in this way the ER acts as a filter at the begining of the secretory

pathway, preventing invalid proteins from progressing further and reaching their destinations in a form that may have a detrimental effect on cellular processes (Hurtley and Helenius, 1989).

Correctly folded nascent glycoproteins leave the ER and travel through the various distal compartments of the secretory pathway. In mammalian cells some of the high mannose chains formed in the ER are processed to complex oligosaccharides which may contain additional GlcNAc residues as well as a variable number of galactose, sialic acid and fucose residues (Kornfeld and Kornfeld, 1985). This processing involves further trimming of the original oligosaccharide added in the ER as well as the addition of further sugars. The oligosaccharide chains linked to Asn residues in yeast proteins are also modified after they have left the ER, being extended by the addition of many more mannose residues to form an outer chain structure which may contain upwards of fifty mannose residues. It is in the Golgi complex of mammalian cells that proteins receive their O-linked oligosaccharide chains (Niemann *et al.*, 1982: Johnson and Spear, 1983), a modification carried out by a series of glycosyl transferases which catalyse the addition of one sugar residue at a time to a protein (up to about ten).

The processes involved in the formation of N-linked oligosaccharide chains are summarised in Figure 1-2a, and Figure 1-2b highlights the differences between the N-linked glycans added to proteins by mammalian cells and by yeast cells.

Figure 1-2

N-linked glycosylation

Figure 1-2a is a diagrammatic representation of the events which occur in the secretory pathway leading to the N-linked glycosylation of proteins as described in the text.

(1) During translocation of the polypeptide across the ER membrane an oligosaccharide moiety is transferred from dolicholpyrophosphate (Dol-P-P) and then processed prior to the protein leaving the ER: three glucose molecules and two mannose molecules are removed by the sequential action of α-glucosidase I (2) , α-glucosidase II (3) and 1,2-mannosidase (4). The oligosaccharide chain undergoes further sequential modification during transit through the Golgi apparatus. In the *cis* Golgi oligosaccharide side chains are trimmed by the removal of four mannose residues by α-mannosidase I (5). A molecule of N-acetyl glucosamine is added by N-acetyl glucosaminyltransferase I (6) in the medial Golgi, an event which is followed by the removal of one more mannose residue by α-mannosidase I (7). Finally in this compartment, a second molecule of N-acetyl glucosamine and a molecule of fucose are added by N-acetyl glucosaminyltransferase II (8) and fucosyltransferase (9) respectively. In the *trans* cisternae of the Golgi complex two molecules of galactose are added by the action of galactosyltransferase (10) and this is followed by the addition of two molecules of sialic acid by sialyltransferase (11).

Figure 1-2b highlights the differences between the N-linked oligosaccharide chains attached to the glycoproteins of mammalian cells and yeast cells. Also shown is the site of action of endoglycosidase H (endo H).

Figure 1-2a The biogenesis of N-linked glycans

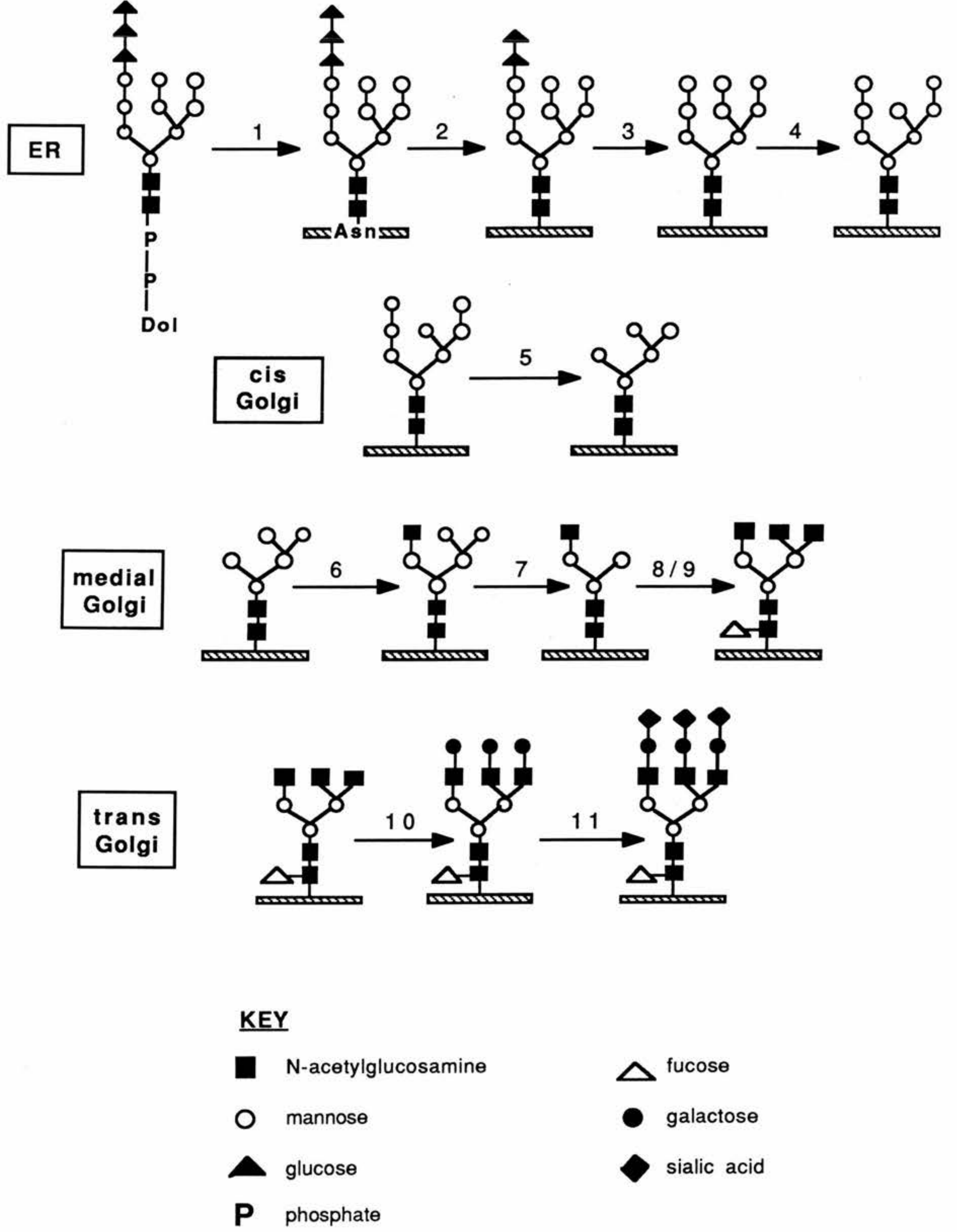
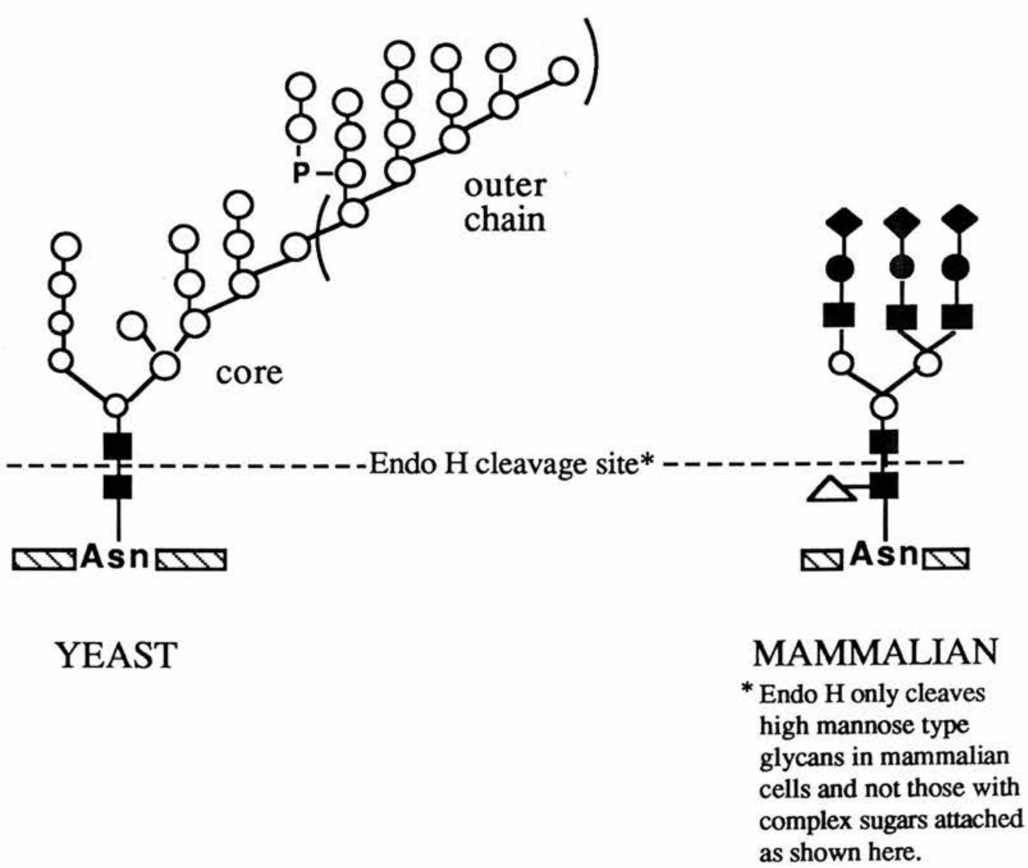


Figure 1-2b
A comparison of N-linked glycosylation in yeast and mammalian cells

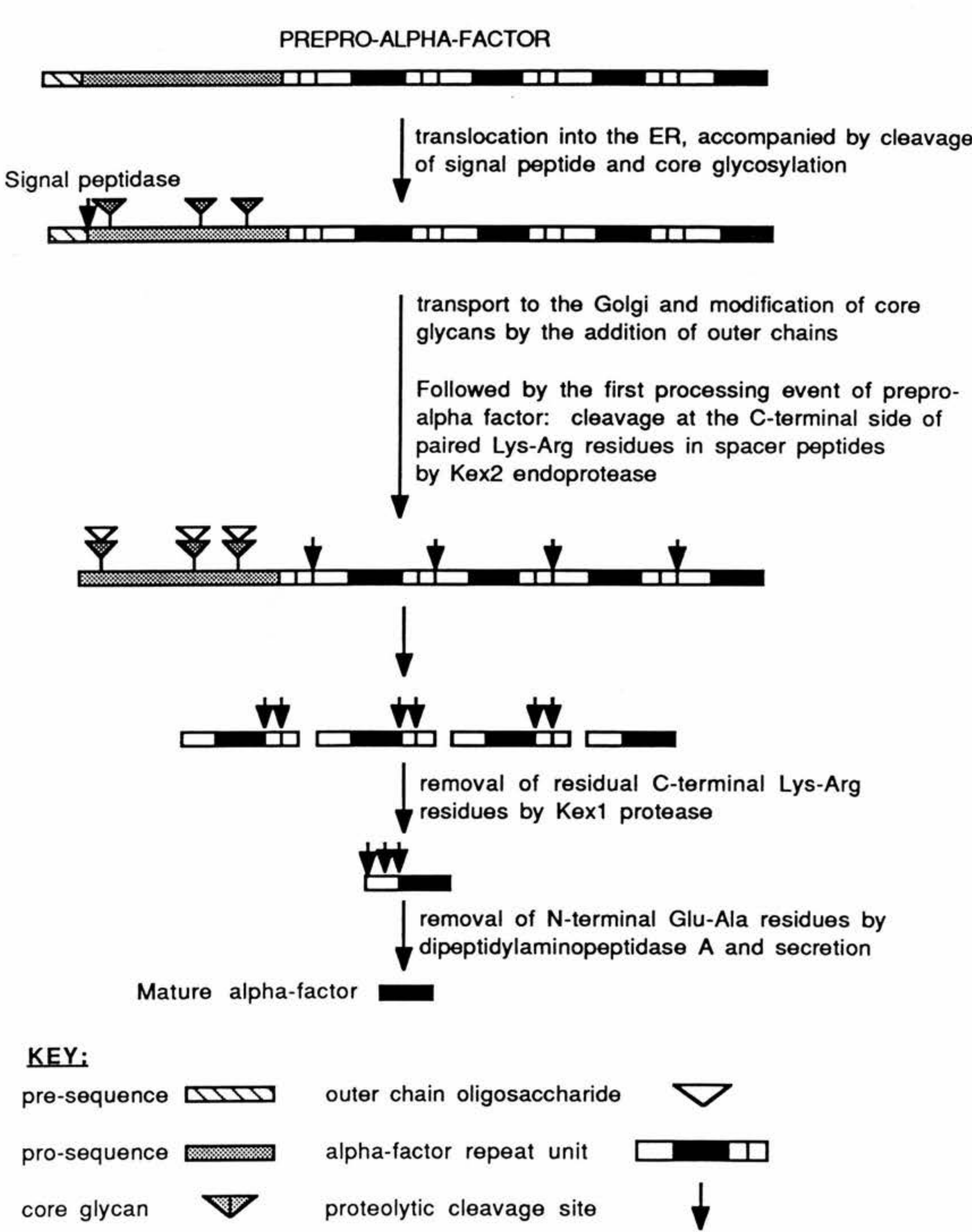


The containment of degradative enzymes within the lysosome or vacuole was cited earlier as an example of the importance of compartmentalisation in eukaryotic cells. Proteins are often synthesised in an inactive form subject to activation as required in order to prevent inappropriate functioning of the protein. One way in which a cell can convert an inactive polypeptide to its active form is by the use of proteolytic processing (Thomas *et al.*, 1988). An example of this is evident in the synthesis of the yeast mating pheromone α-factor. Yeast cells of mating type α secrete the peptide α-factor which interacts with cells of mating type **a**, and is involved in the initiation of diploid formation (for review see Herskowitz, 1986). The pheromone is synthesised as an inactive precursor molecule, prepro-α-factor, and the removal of a signal peptide in the ER generates pro-α-factor which is also inactive (Waters *et al.*, 1988). Complete maturation of pro-α-factor to the secreted thirteen residue peptide requires cleavage of the precursor at the carboxyl side of lys-arg sites followed by the removal of N and C terminal residues from the peptides yielded by these proteolytic cleavages. The primary cleavages occur after the peptide has left the ER and is carried out by the product of the *KEX2* gene, a Ca^{2+}-dependent, neutral serine protease (Julius *et al.,* 1984). Subsequent removal of of the C-terminal basic residues is achieved by the action of the *KEX1* gene product (Dmochowska *et al.*, 1987: Achstetter and Wolf, 1987), with the product of *STE13* being responsible for the removal of an N-terminal spacer through its action as a dipeptidyl aminopeptidase (Julius *et al.*, 1983). The various events involved in the biosynthesis of α-factor were elucidated at least in part with the aid of *sec* mutants (Julius *et al.*, 1984) and are summarised in Figure 1-3 (Fuller *et al.,* 1988).

Figure 1-3

Processing of prepro-α-factor

Shown here are the post-translational processing events involved in the maturation of the product of the *MFα1* gene product, prepro-α-factor. The order of these events was elucidated by using biochemical and immunocytochemical identification of various precursor forms of the mature protein (α-factor) and by the analysis of individual processing enzymes and their genes (see text for details, adapted from Fuller *et al.*, 1988).



The various processing events to which a protein may be subjected as it travels through the secretory pathway are carried out in a strictly defined order. This sequence of events has been identified (Palade, 1975) using pulse-chase labelling, autoradiography and membrane fractionation work and can be used to determine at which stage in the pathway a particular protein is at any one time. Investigation of the glycosylation state of a protein, for example, can be used to gain such information (Esmon *et al.,* 1981). Due to the variability in the size and number of outer chain units added to yeast glycoproteins after they have left the ER there is marked heterogeneity in their final molecular weight. It is because of this that gel electrophoresis of such proteins results in broad smears. Gel electrophoresis of the same protein which had not, for some reason, progressed from the ER would result in a more defined band representing the protein whose glycosylation state at this stage in its existence is more homogeneous, consisting of the core oligosaccharide chain. It is possible to convert the 'broad smear' form of such proteins to a homogeneous form by treating them with the enzyme endoglycosidase H (Trimble and Maley, 1984) which removes most of the carbohydrate moiety (see Figure 1-2b). Such approaches can be used to determine in which organelle a protein is at any one time, and have been used to study the mechanisms by which proteins are transported between different compartments of the secretory pathway in transport assays.

2.3. Identification and characterisation of NSF/Sec18p

By monitoring the covalent modification of a protein known to occur as it is transported from one compartment of the mammalian Golgi to the next (Fries and Rothman, 1980), Rothman and co-workers identified a protein that is necessary for this transport step. Cells infected with vesicular stomatitus virus (VSV) synthesise the virally encoded G-protein (a viral coat glycoprotein) in large amounts. This membrane protein enters the host's secretory pathway and is subjected to post translational modifications as it travels through the various compartments of the pathway to the plasma membrane. VSV-G-protein has been used in a number of transport assays and is useful in such studies

especially when the donor and acceptor compartments are prepared from different cells as it can be assured that only the former will contain the protein by preparing it from cells infected with the virus while the acceptor fraction is prepared from uninfected cells. Reconstitution of membrane transport between successive cisternae of the Golgi complex in mammalian cells revealed that the process was energy dependent since the delivery of VSV-G-protein from a Golgi-enriched donor fraction prepared from a VSV-infected mutant Chinese hamster ovary (CHO) cell line lacking the glycosyltransferase N-acetyl glucosaminyltransferase I to an acceptor Golgi fraction prepared from uninfected wild-type CHO cells requires not only cytosol, but also a supply of energy in the form of ATP. Membrane transport from the *cis* compartment of the donor fraction to the N-acetyl glucosaminyltransferase-containing medial cisternae of the acceptor was shown to have taken place when the two fractions were mixed together in the presence of cytosol, an ATP regenerating system, and also UDP-(^{3}H)-GlcNAc (a substrate for the N-acetyl glucosaminyltransferase), by demonstrating that, after incubation at 37°C, label was incorporated into VSV-G-protein (Balch *et al.*, 1984: Braell *et al.*, 1984). The fact that this transport step could be inhibited by treating the reaction mixture with the sulphydryl alkylating agent *N*-ethyl maleimide (NEM) prior to incubation led to the identification and subsequent purification of NEM-sensitive factor (NSF), a homotetramer of 76K subunits (Block *et al.*, 1988).

Since its discovery NSF has been shown to be required for a number of membrane traffic events including the delivery of VSV-G-protein from the ER to the Golgi in MDCK (Madin Darby canine kidney) cells, an event which can be followed by monitoring the formation of the form of the protein carrying five mannose residues (a product of one of the modification events associated with the Golgi complex), in semi intact cells (Schwaninger *et al.*, 1991). This delivery is sensitive to NEM, as well as to an anti-NSF antibody (Beckers *et al.*, 1989). Further evidence that the same protein is involved in the two separate protein transport reactions comes from the fact that the block introduced by NEM on ER to Golgi transport is reversible by the addition of NSF purified using the intra-Golgi transport system.

Studies involving NSF from mammalian cells have been elegantly complemented by genetic studies using yeast cells and these have furthered our knowledge concerning the role of this protein in protein transport. Yeast cells bearing a temperature sensitive mutation in the *SEC18* gene accumulate vesicles associated with the ER as well as intracellular pools of secretory proteins bearing characteristic ER modifications at 37°C (Novick *et al.*, 1981). The protein product of *SEC18* has 48% sequence similarity with NSF (Wilson *et al.*, 1989), a finding which was initially surprising since at that time NSF had not yet been shown to be required for transport between the ER and the Golgi in mammalian cells, and *sec18* mutants were only known to be defective in the transport of proteins from the ER. A cytosolic fraction prepared from yeast cells can replace NSF in the CHO intra-Golgi transport system, but cytosol prepared from *sec18* cells lacks this ability (Wilson *et al.*, 1989). The belief that the two proteins carry out the same function gained further backing from the fact that cytosol prepared from yeast cells overexpressing *SEC18* exhibits enhanced NSF activity. It was this homology, between a protein required for the intra-Golgi transport of proteins and one required for transport from the ER, that led to the idea that the same factor might be required at multiple stages in the secretory pathway.

Cells carrying a temperature sensitive *sec18* allele fail to transport secretory proteins past the ER at 37°C, but there is evidence that Sec18p is also required in subsequent stages of the yeast secretory pathway. This was shown by taking advantage of the rapid thermal inactivation of Sec18p. The secretory pathway of a temperature sensitive *sec18* strain was preloaded with radioactively labelled proteins prior to the introduction of the *sec* block, following which the glycosylation states of the intracellular pool of secretory proteins were investigated (Graham and Emr, 1991). The pool was found to contain not only proteins bearing oligosaccharides characteristic of residency in the ER, but also proteins which had been modified to various extents by processes known to occur after proteins have left this organelle (including proteins in their mature forms). Since these proteins had not been secreted, and could be located to more than one

intracellular compartment by virtue of modifications they had received, it was deduced that, as would have been expected from its homology with NSF, in addition to its role in the transport of proteins from the ER, Sec18p is required in subsequent stages of the yeast secretory pathway. A suprising finding from this work is that Sec18p is not required for transport of proteins to the vacuole.

It is noteworthy that *sec18* mutants exhibit defects in endocytosis (Riezman, 1985), and also that NSF has been shown to participate in the fusion of mammalian endocytotic vesicles with endosomes *in vitro* (Diaz *et al.*, 1989). One assay used to demonstrate this involves two different ligands which bind to the same receptor located in the plasma membrane of macrophages (Diaz *et al.*, 1989). A DNP (di-nitro phenol) derivative of β-glucuronidase was incubated with, and subsequently bound to, the mannose receptor of one population of macrophages, while an anti-DNP antibody was similarly bound to the same receptor on a second population of the cells. The two cell populations were homogenised separately, and post nuclear supernatants prepared from these homogenates were mixed together. The formation of membrane bound antibody-antigen complex was followed as a measure of endocytotic fusion. Such fusion was shown to require cytosol and ATP, as well as NSF.

2.4. NSF/Sec18p functions in association with other proteins

In order to achieve maximum NEM inhibition of protein transport in the systems described it was found to be necessary to treat both membrane and cytosol fractions with the reagent (Malhotra *et al.*, 1988). From this finding it was deduced that NSF exists in both soluble and membrane associated forms, consistent with the fact that Sec18p exists in both soluble and membrane associated forms within yeast cells (Eakle *et al.*, 1988). The C-terminal two thirds of the protein consists of two repeats characteristic of ATP binding sites (Wilson *et al.*, 1989). Mutations in either of these two regions reduces the ATPase activity of the protein and destroys its fusion activity demonstrating that ATP hydrolysis plays an important role in the protein's ability to

stimulate membrane fusion (Rothman and Orci, 1992). The sequence of the 76K polypeptide subunit of NSF suggests that it is a soluble cytosolic protein, lacking any obvious hydrophobic regions as are found in viral proteins that induce membrane fusion (Wilson *et al.*, 1989). This may indicate that, rather than it itself being a 'fusogen', NSF promotes some other, as yet unknown, factor to stimulate membrane fusion, and that its membrane association is facilitated by interaction with some other component(s).

The active component involved in membrane fusion is believed to be a 20S particle (Wilson *et al.*, 1992), consisting not only of NSF, but also α, β and γ SNAPs or NSF attachment proteins (Clary *et al.*, 1990). It is this complex that is believed to bind to integral membrane receptors present in the acceptor compartment (Weidman *et al.*, 1989). The three SNAPs, water soluble proteins (35K, 36K and 39K) are unable to bind NSF in solution but can do so when bound to a membrane receptor (Goda and Pfeffer, 1989). An integral membrane protein has been identified as a SNAPs receptor in mammalian Golgi membranes and since it has been demonstrated that α- and β–SNAPs compete for binding to membranes it is likely that they share the same receptor and have similar functions. γ-SNAP appears to bind to its own separate receptor and is thought to have a function distinct from that carried out by α– and β-SNAPs (Wilson *et al.*, 1992). Hydrolysis of ATP by NSF results in the disassembly of the 20S particle (Wilson *et al.*, 1992) which, since the ATPase activity of NSF is required for membrane fusion, is likely to be related to membrane fusion.

sec17 cells are defective in the transport of proteins from the ER and accumulate 50nm vesicles (Kaiser and Schekman, 1990). The product of the *SEC17* gene has been shown to be functionally equivalent to α-SNAP (it can replace the mammalain protein in a transport assay) and is found in a complex which also contains Sec18p (Griff *et al.*, 1992). It has been stated in a prominent review that Sec17p is required not only for transport of proteins from the ER, but at multiple stages throughout the yeast pathway (Rothman and Orci, 1992). Such a finding would indeed provide evidence that

α-SNAP may serve to attach NSF to membranes at various stages in the secretory pathway, but the reference cited in the review to back up this statement does not show that Sec17p functions at multiple stages throughout the yeast secretory pathway (Graham and Emr, 1991) and therefore some confusion exists in this area. It is from results such as those discussed so far that many elaborate models explaining how the various components identified as being involved in membrane fusion may interact have been proposed. While such models can be useful it is important to remember that they are only models and that they must be adapted as new results demand and that they alone should not be used to cast doubt on any data which question their validity.

2.5. GTP-binding proteins are involved in membrane traffic events

The yeast genes *SEC4* and *YPT1* encode proteins that are required for the fusion of secretory vesicles with the plasma membrane and the transport of proteins from the ER respectively (Novick *et al.*, 1981: Segev *et al.*, 1988). The discovery that both Sec4p and Ypt1p are homologues of the GTP-binding, transforming protein ras (Gallwitz *et al.*, 1983: Salminen and Novick, 1987: Wagner *et al.*, 1987: for a review of the ras superfamily see Bourne *et al*, 1990, and also Valencia *et al.*, 1991) led to an investigation into the role of GTP-binding proteins in membrane trafficking events (for a recent review on this field see Pfeffer, 1992 and also Gruenberg and Clague, 1992). *In vitro* transport of VSV-G-protein in the two mammalian systems that were described earlier (Section 2.3) is inhibited by the non hydrolysable analogue of GTP, GTPγS as well as by NEM (Melançon *et al.*, 1987). The inhibition exerted by GTPγS leads to the accumulation of coated vesicles, and coated buds are evident on Golgi membranes (Melançon *et al.*, 1987). NEM inhibition on the other hand leads to an accumulation of uncoated vesicles (Malhotra *et al.*, 1988). It is evident that the GTPγS block precedes that imposed by NEM since the presence of both inhibitors results in an accumulation of coated vesicles of the type associated with the GTPγS block (Orci *et al.*, 1989). These observations have led to a model of membrane traffic (Figure 1-4) in which

coated membrane vesicles bud from the donor compartment and are uncoated in an energy requiring process before docking on to, and finally fusing with the acceptor membrane. It is in this final fusion event, a Ca^{2+}-dependent process which causes the vesicles to deliver their contents to the donor compartment, that NSF was initially believed to be involved (Malhotra *et al.*, 1988: Beckers *et al.*, 1989: but see Section 2.6).

The isolation of coated vesicles from a GTPγS blocked *in vitro* protein transport system (set up using mammalian components) led to the identification of cytoplasmically disposed proteins believed to be responsible, at least in part, for the formation of coated vesicles from donor membranes during secretion (Malhotra *et al.*, 1989; Serafini *et al.*, 1991a). These coat proteins (COPs) which are present in coated vesicles in stoichiometric proportions, include α, β, γ and δ-COP as well as the small GTP-binding protein ARF (Serafini *et al.*, 1991b). Immunocytochemistry has been used to show that, as well as being associated with the coated vesicles defined by the GTPγS block, β-COP and ARF both exist in cytoplasmic forms but are not associated with the vesicles that accumulate upon inactivation of NSF (Duden *et al.*, 1991b; Orci *et al.*, 1991b). Many models, in trying to explain the mechanics of membrane traffic events, invoke the association and dissociation of various complexes such as the formation of vesicle coats from their constituents (Waters *et al.*, 1991). Models of this type are attractive since they introduce a number of levels at which events can be controlled. Such a consideration is likely to be important in a system where insufficient control could result in incorrect fusion which could have drastic results. The apparent absence of any of the COPs from Golgi membranes (except concominant with bud formation) has led to the idea that the coats observed on the vesicles are assembled from cytosolic subunits prior to budding and the presence of β-COP and ARF in the cytosol supports this idea.

It was mentioned in Section 2.1 that the 'ER-blocking' *sec* mutants can be divided into two classes (Kaiser and Schekman, 1990); those that accumulate 50nm vesicles, and

those that do not (classes II and I respectively). Whereas mutations in the class II genes (*SEC18, 17* and *22*) inhibit the fusion of the 50nm vesicles with their target organelle, mutations in the class I genes, *SEC12, 13, 16, 21* and *23*, prevent the budding of membrane from the donor organelle to form the vesicles. The gene products of both *SEC13* and *SEC23* are involved in complexes found in the cytosol (Hicke and Schekman, 1989: Pryer *et al.*, 1990: Hicke *et al.*, 1992) and it is thought (Rothman and Orci, 1992) that they may be components of a coat similar to that observed in mammalian systems (although no such coat has been observed in yeast due to the limited use of electron microscopy with this organism). However, the mammalain homologue of Sec23p is restricted to a cytoplasmic zone between the ER and the Golgi complex in mammalian cells (Orci *et al.*, 1991a) suggesting that its role is confined to vesicle budding from the ER. The 50nm vesicles associated with the *sec18* block (and that imposed by mutations in *SEC17* and *SEC22*) are predicted to be uncoated since they are accumulated as a result of the equivalent of the NEM block in mammalian cells (Rexach and Schekman, 1991).

Since NSF/Sec18p has been found to be required for a number of different membrane transport steps it is unlikely to be involved in controlling the specificity of membrane fusion. Such specificity is of central importance to the secretory pathway: to ensure that proteins travelling along it are correctly processed; to ensure that proteins are accurately targetted as required; and to prevent random membrane fusion events which, if allowed to occur, could prove catastrophic to the cell. The identification of a number of *YPT1/SEC4*-like genes in mammalian systems (Zahraoui *et al.*, 1989: Chavrier *et al.*, 1990), many of whose products have been localised to distinct compartments, and the fact that GTPγS inhibits multiple stages of the secretory pathway (Melançon *et al.*, 1987: Tooze *et al.*, 1990: Rexach and Schekman 1991: D'enfert *et al.*, 1991) support the proposal that each step of membrane transport is controlled, at least in part, by a protein belonging to the ras superfamily. It has been suggested that these GTP-binding proteins (or rab proteins) ensure that transport vesicles fuse only with the appropriate membrane compartment using a mechanism similar to that used by EF-Tu as it ensures

codon-anticodon fidelity (Bourne *et al.,* 1990). GTP-binding proteins operate in many cellular systems as molecular switches. These proteins adopt different conformations (and thus have different activities) depending on which nucleotide they have bound (GTP or GDP). EF-Tu binds tightly to amino acyl-tRNAs in its GTP bound state, and codon recognition by the tRNA triggers GTP hydrolysis. With GDP bound the factor is no longer able to participate in the complex and is released leaving the tRNA bound to the ribosome. This process prevents the misincorporation of amino acids into a growing polypeptide chain since only a correct codon-anticodon match will bind the EF-Tu-amino acyl tRNA complex to the ribosome for a sufficient period of time, until the tRNA is released by EF-Tu.

Perhaps one of the most direct lines of evidence for the involvement of a member of the ras superfamily in membrane fusion events comes from observations that the dissociation of rab3A from synaptic vesicles correlates with exocytosis of glutamate from synaptosomes *in vitro* (Fischer von Mollard *et al.,* 1991). Conversely, repolarisation of these membranes leads to the reassociation of rab3A with the vesicles, an association mediated by a post-translational covalent modification of the protein. This demonstration of the reversible dissociation of a GTP-binding protein from membranes undergoing fusion supports models which suggest that the cycling of such proteins between membrane bound and cytosolic states offers control over membrane fusion (Pfeffer, 1992).

Subcellular fractionation has determined that 15% of Sec4p forms a cytosolic pool in wild type cells, with most of the other 85% being associated with the plasma membrane and some with secretory vesicles (Goud *et al.,* 1988). The membrane association of Sec4p is characteristic of it being an integral membrane protein and since *SEC4* doesn't encode a signal peptide, or any obvious hydrophobic domains which could serve to anchor the protein in the lipid bilayer it is believed that Sec4p undergoes a post translational modification in order to acheive its membrane association. Ras proteins are subjected to the addition of a isoprenyl unit to a Cys residue found within the C-

terminal sequence Cys-Cys, Cys-X-Cys, Cys-Cys-X-X or Cys-Cys-X-X-X; where X represents any residue (Farnsworth *et al.*, 1991: Khosravi-Far *et al.*, 1991). Sec4p has the C-terminal sequence of Ser-Asn-Cys-Cys which may serve the same function as the Cys containing sequences found in rab proteins. Ypt1p has the C-terminal sequence Gly-Gly-Cys-Cys and it has been shown that the Cys residues within this sequence are essential for its function (Molenaar *et al.,* 1988). The gene *BET2* was initially identified as being required for transport of proteins from the ER of yeast cells (Newman and Ferro-Novick, 1987). Its product is required for the membrane attachment of Sec4p and Ypt1p, a fact demonstrated by the observation that there is an increase in the soluble pools of these proteins in cells bearing a mutation in *BET2* (Rossi *et al.*, 1991). Bet2p is homologous to Ram1p, a component of a protein prenyltransferase that modifies ras allowing its membrane attachment (Kohl *et al.,* 1991). Although no isoprenylation of either Sec4p or Ypt1p has been detected it is still believed that such a modification is responsible for their membrane attachment. This theory is supported by evidence which shows that mutant cells in which the synthesis of the isoprenoid precursor mevalonic acid is blocked have an increased pool of soluble Sec4p (Rossi *et al.*, 1991). The failure to detect isoprenylation of either Sec4p or Ypt1p may be due to technical difficulties in that *S. cerevisiae* does not incorporate externally added mevalonic acid into proteins. Recent studies have demonstrated that the fission yeast *Schizosaccharomyces pombe* will incorporate label into several proteins (20-26kD) following incubation with tritiated mevalonic acid (Giannakouros *et al.*, 1992) and such work may lead to the demonstration of isoprenylation of small GTP-binding proteins in yeast.

Reconstitution of intra Golgi transport of VSV-G-protein *in vitro* requires fatty acyl CoA (Pfanner *et al.,* 1990). Palmityl CoA is required after coated vesicles have budded from the donor membrane, moved to the acceptor membrane, uncoated and bound NSF (as depicted in Figure 1-4). It is tempting to speculate that fatty acyl CoA is required in order to acylate some component which could then promote membrane fusion by, for example perturbing the lipid bilayers or anchoring other fusion components in the

membrane. Palmitate binds to proteins via a thioester or oxyester linkage both of which can be easily hydrolysed by specific esterases (Schultz *et al.,* 1988). It can be envisaged that the control of acylation and deacylation of a component would offer a level of control of membrane fusion events. The budding of coated vesicles also requires fatty acyl CoA (Pfanner *et al.,* 1989) and thus it is thought that proteins with a covalently bound hydrophobic moiety may play a crucial role in both the budding of transport vesicles, and in their fusion with the relevant acceptor membrane. The small GTP-binding protein ARF found in the 20S NSF-containing particle is N-terminally myristylated (Kahn *et al.,* 1987) and is required for the budding of vesicles from Golgi membranes. Myristylated ARF with GTP bound is capable of inserting into lipid bilayers but the absence of GTP strips the protein of this ability (Kahn *et al.,* 1987). One theory of ARF's involvement in budding suggests that, as it binds GTP, ARF inserts into the membrane of the donor organelle allowing COPs to form the coat complex around it and anchors them in the membrane once the coat is fully formed (Rothman and Orci, 1992). GTP hydrolysis occuring on encounter with the target membrane would destabilise the coat and dissociate it from the membrane, recycling its constituents to the cytosol.

Whereas the myristylation of ARF and the prenylation of rab proteins may explain their membrane association, they do not explain their organelle specific distributions. Rab protein sequences are most divergent in their C-terminal sequences and it has been shown that the location of rab5 can be changed to that of rab7 by transplanting the C-terminal thirty four amino acids of the latter onto the former (Chavrier *et al.*, 1991). Overexpression of rab proteins does not lead to their mislocalisation but to their cytoplasmic accumulation (Gorvel *et al.*, 1991) an observation which is consistent with the existance of saturable rab receptors suggesting that the specificity of rab localisation may be achieved in this way.

Early endosome fusion detected in an assay similar to that described above (Section 2.3) has been found to be sensitive to GTPγS and can be inhibited by the addition of cytosol prepared from a mutant cell line which overexproduces a mutant form of rab5 (that is unable to bind GTP). The fusion is stimulated by cytosol prepared from cells overexpressing wild type *RAB5* and is specifically inhibited by the addition of anti-rab5 antibodies (Gorvel *et al.,* 1991). The specificity of these small GTP-binding proteins is demonstrated by the fact that antibodies against other members of the same family of proteins do not impare the function of rab5 in endosome fusion. Similar work has been carried out using rab1b which has been localised to the ER and the Golgi. Antibodies against this ras homologue inhibit transport between these two compartments in semi-intact cells (Plutner *et al.*, 1991). The time at which these antibodies inhibit this transport is consistent with ras homologues being required for vesicle targetting (similarly antibodies against Ypt1p block transport between the ER and the Golgi after vesicle formation) as is the accumulation of vesicles in yeast cells harbouring *sec4* mutations (Rexach and Schekman, 1991: Segev, 1991).

In addition to vesicle targetting and fusion, GTP hydrolysis appears to be needed for vesicle budding (Beckers and Balch, 1989: Tooze *et al.*, 1990: Goda and Pfeffer 1991), but is not necessary for all vesicle formation processes since GTPγS causes the accumulation of transport vesicles (see above: Melançon *et al.*, 1987). Direct evidence for the involvement of GTP-binding proteins in the formation of transport vesicles has come from work involving a suppressor of *sec12* mutations, *SAR1*. Sar1p is a GTP-binding protein required for transport between the ER and the Golgi (Nakano and Muramatsu, 1989) and the *SAR1* suppression of *sec12* has been reconstituted *in vitro* (Oka *et al.*, 1991) as has a direct biochemical interaction between Sar1p and Sec12p that is required for the formation of transport vesicles from the ER (D'enfert *et al.*, 1991).

There is also evidence for the involvement of heterotrimeric GTP-binding proteins (the type involved in signal transduction at the plasma membrane) in membrane trafficking

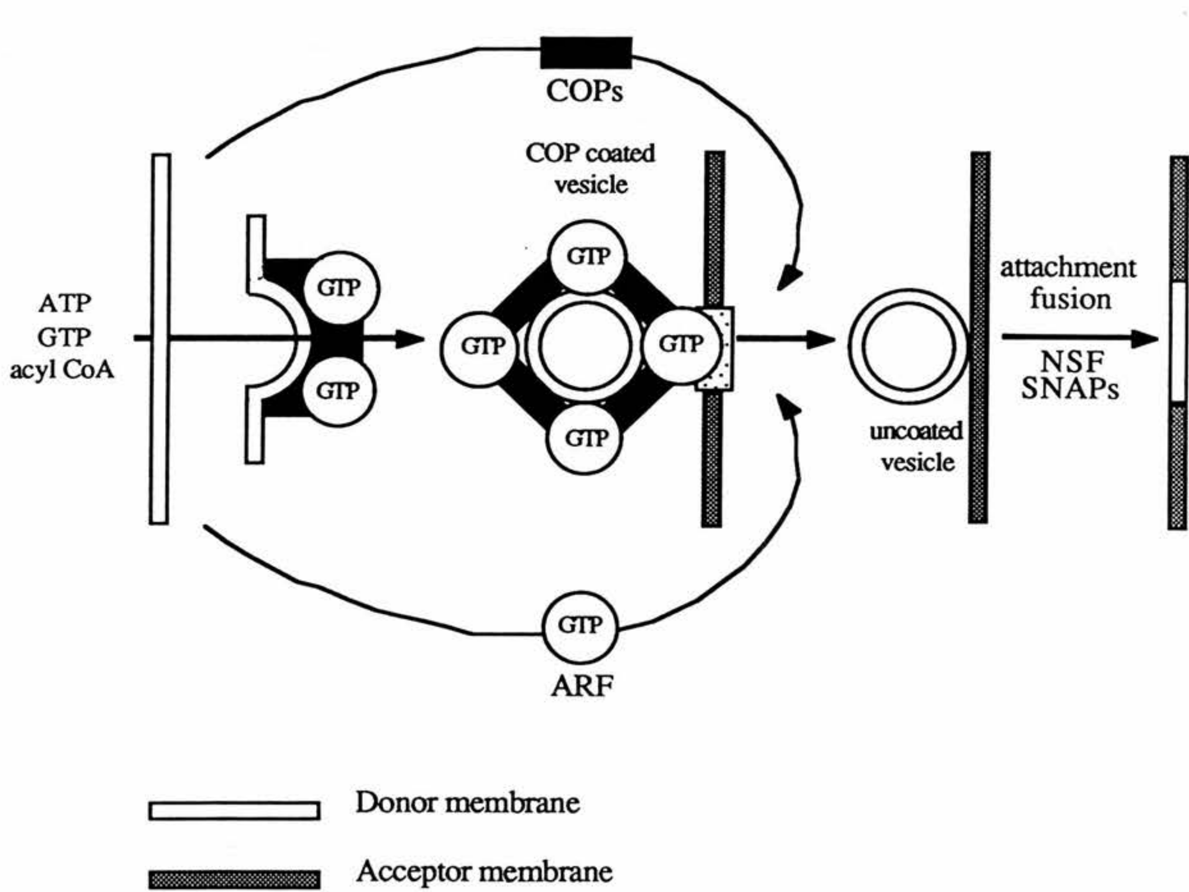
events (for review and proposed models of how such proteins may be involved see Barr *et al.*, 1992 and also Pfeffer, 1992). It has been known for some time that such proteins are found, not only on the plasma membrane, but also on some intracellular membranes, such as the ER (Audigier *et al.*, 1988) and the Golgi complex (Ercolani *et al.*, 1990) suggesting that they may be involved in the regulation of some function(s) of these organelles. The retardation of secretion of proteoglycan from cells treated with pertussis toxin (Stow *et al.*, 1991) and the inhibition of secretory vesicle formation from the Golgi complex by AlF^- *in vitro* (Barr *et al.*, 1991) both suggests that trimeric GTP-binding proteins are involved in these membrane trafficking events (pertussis toxin ADP-ribosylates the α_{i3}-subunit of trimeric G-proteins and AlF^- is known to activate trimeric G-proteins but has no effect on G-proteins of the ras superfamily: Higashijima *et al.*, 1989: Kahn, 1991).

From the above discussion it can be seen that cell free systems in which various stages of the secretory pathway have been examined in isolation have played a crucial role in the identification of factors involved in the pathway. Models of how these components might act in the cell are then proposed, often taking into consideration information gleaned from work utilising yeast mutants, and later modified, hopefully becoming closer to reality, as more and more information about the secretory pathway is gathered.

Figure 1-4

A model of intra Golgi vesicular traffic

Summarised here is a working model for vesicular transport between Golgi compartments proposed by Rothman and co-workers (Rothman and Orci, 1992). The membrane on the left (blank) represents a portion of Golgi membrane from which COP coated vesicles bud. These then fuse with the target membrane (shaded), of the subsequent Golgi compartment. Specific targetting of the coated vesicles is proposed to be initiated by an unknown component present in/on the acceptor membrane. The vesicle is then uncoated and the NSF-dependent fusion pathway is triggered. Components of the coated vesicle include a complex of COPs (black squares) and ARF which can exist in both GTP- and GDP-bound forms.



2.6. Reconstitution of protein transport within the yeast system

Experiments involving the reconstitution of pro-α-factor transport from the ER to the next stage in the yeast secretory pathway (Baker *et al.,* 1988) demonstrate how the genetic approach used to study secretion in yeast has been used in conjunction with a biochemical/cell biology approach similar to that used to study the same system in higher eukaryotic cells.

Messenger RNA can be translated in yeast lysates to generate a 19K form of prepro-α-factor, and it is possible to use such a system to produce ^{35}S-met-labelled prepro-α-factor which can be used to study protein transport. The labelled precursor can be translocated post-translationally into either yeast microsomes or the ER of permeabilised spheroplasts resulting in the protein being core glycosylated and yielding a radioactive protein with an apparent molecular weight of 30K. The transport of core glycosylated pro-α-factor to the next compartment of the yeast secretory pathway can be followed by monitoring the formation of a form of the protein that migrates slowly in gel electrophoresis and is recognised by α-1,6 mannose specific antibodies. The formation of this more heavily glycosylated pro-α-factor from the 30K form of the protein requires the addition of ATP, cytosol and membranes to a system containing the core glycosylated form sequestered in the ER. The addition of outer chain carbohydrate was found to be accompanied by the movement of the radiolabelled protein from rapidly sedimenting membranes to a population that sediment more slowly and which are depleted of NADPH-cytochrome *c* oxidoreductase (an ER marker). This was one criterion that was used to demonstrate that protein transport was being observed rather than the fusion of two compartments, a phenomenon which may have given similar results. The fact that GTPγS inhibits the appearance of the form of the protein recognised by the α-1,6 mannose antibodies but not the formation of the 30K form also provides evidence that two separate events are being followed here, and that transport of the protein between the two compartments involves a GTP binding protein. The transport system is also inhibited by NEM suggesting the involvement of Sec18p at this stage in the pathway.

This cell-free system was established using wild type yeast cells, but has also been used to demonstrate that the transport could be detected between membranes prepared from *sec23* cells (which are deficient in the transport being measured) if they were supplemented with cytosol prepared from *SEC23* (wild type) cells (cytosol from *sec18* cells also failed to support the reaction in agreement with the block imposed by NEM). *SEC23* encodes an 84K protein but the above system has been used to demonstrate that it exerts its effects on the acceptor compartment of the reaction by associating with the cytoplasmic face of the membrane as part of a 400K complex (Hicke and Schekman, 1989). This demonstrates the power of *in vitro* transport assays to aid the understanding of the molecular mechanisms involved in protein transport.

Standard genetic techniques were used to identify and isolate *BOS1* (Newman *et al.*, 1990), a gene which has been shown to suppress mutations in two genes which disrupt the above transport system (*SEC22* and *BET1*). Because of its ability to suppress these mutations it was thought that the product of this gene must function in the transport of proteins from the ER and the ability to follow the transport of pro-α-factor as it travels through the yeast secretory pathway has provided evidence to support this.

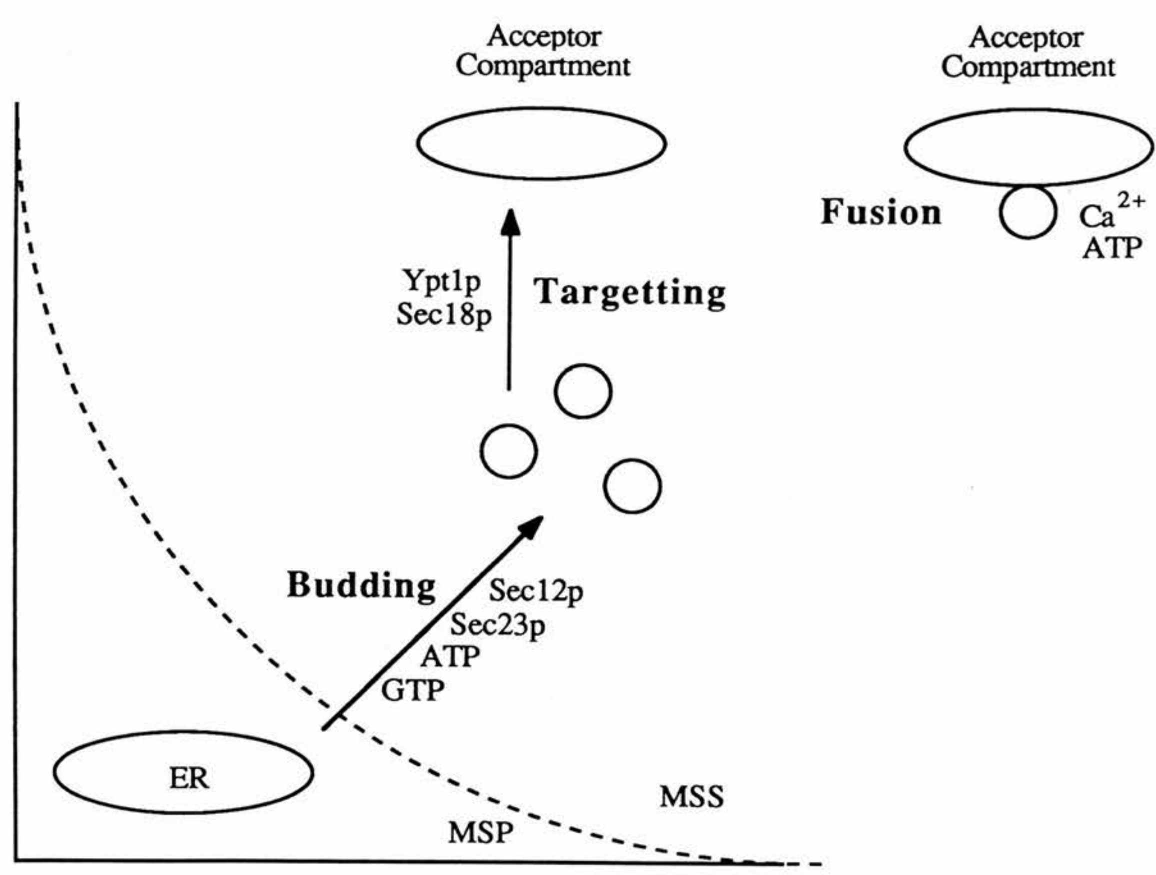
Transport of pro-α-factor from the ER as described above has been divided into three separate events that can be followed in the assay (Rexach and Schekman, 1991): (1) vesicle budding from the ER; (2) targetting of these vesicles to the acceptor membrane; and (3) fusion of the transport vesicles with the acceptor membrane. Vesicle budding from the ER can be followed by monitoring the appearance of slowly sedimenting membranes (which fractionates to a medium speed supernatant or MSS) containing core glycosylated pro-α-factor (the ER, as 'characterised' by the marker enzyme NADPH-cytochrome *c* oxidoreductase sediments rapidly). Vesicle targetting was followed by monitoring the sedimentation of core glycosylated pro-α-factor in a sucrose density gradient to the same fraction as membranes containing outer chain glycosylated pro-α-factor (it had been shown that the transport vesicles containing core glycosylated pro-

α-factor sedimented to a different fraction from the highly glycosylated form). Vesicle fusion was followed by monitoring the conversion of core glycosylated pro-α-factor to the highly glycosylated form of the protein in a MSS. The differential inhibition of these three stages involved in the transport reaction by mutant *SEC* gene products and chemical inhibitors has led to the model shown in Figure 1-5 to be proposed. The way in which this system has been used demonstrates how genetics and cell biology can work together to identify and characterise components involved in protein transport. The results depicted in Figure 1-5 suggest that Sec18p is not involved in the actual membrane fusion event, but rather serves as a docking protein to bring the two membranes into close proximity since the ER to Golgi transport defect displayed by yeast *sec18* mutant lysates is apparently restricted to targetting. The Ca^{2+} dependent vesicle fusion intermediate can be produced in a *sec18* lysate at the permissive temperature and can be used to show that fusion proceeds at both 25°C and 37°C upon addition of Ca^{2+} and ATP (Rexach and Schekman, 1991). This has been taken to indicate that Sec18p may not participate directly in membrane fusion (Schekman, 1992) as discussed in Section 2.5. However, these findings do not rule out the possiblity that Sec18p participates in the fusion reaction since it is possible that once assembled into a fusion-competent form the fusion activity of the mutant form of Sec18p may not be thermosensitive, but its ability to assemble into such a form is. Such a scheme fits with recently reported results which demonstrate that, although required for the formation of functional vesicles (in an assay similar to that used by Balch *et al.*, 1984), the NEM sensitive function of NSF is not required for the attachment of these vesicles to the acceptor compartment or their subsequent fusion with the acceptor membrane (Wattenberg *et al.*, 1992). If NSF does have a role in membrane fusion, as discussed in Section 2-5, these results indicate that it is incorporated into vesicle membranes during vesicle formation and carried to the site of membrane fusion in this way.

Figure 1-5

A yeast cell free transport system

Depicted here is a breakdown of transport of pro-α-factor from the ER in yeast cells to the next compartment in the secretory pathway. Inhibition of various stages of the transport of pro-α-factor by mutant *sec* gene products and chemical inhibitors in permeabilised spheroplasts has led to the formation of the following model explaining how a number of components may function in protein transport (MSS/P; medium speed supernatant/pellet, see text for details, adapted from Rexach and Schekman, 1991).



Section 3. The Golgi complex

3.1. Introduction

The structure and distribution of the organelle first described by Camillo Golgi at the end of the last century (Golgi, 1898) has been described in many eukaryotic cell types (for review see Mellman and Simons, 1992). A text book description of the morphology of the Golgi complex might talk of a system of distinct but interconnected, flattened, membrane-bound compartments (sacs or cisternae), located close to the nucleus of the cell. This organelle is apparent throughout the cell cycle, except in premitotic cells where it appears to fragment prior to distribution between the two daughter cells (Lucocq *et al.*, 1987). Although stacked cisternae can be seen in the fission yeast *S. pombe* (Chappell and Warren, 1989), no such structure is visible in *S. cerevisiae* (Redding *et al.*, 1991), but there is evidence, some of which is discussed below, for a compartment of the yeast secretory pathway believed to be analogous to the mammalian Golgi apparatus.

In mammalian cells the Golgi complex or apparatus is commonly thought of as being made up of at least three sub-compartments each containing their own sets of unique enzymes (Dunphy and Rothman, 1983: Duden *et al.*, 1991b). Although immunocytochemistry provides evidence for this (Roth and Berger, 1982: Slot and Geuze, 1983: Roth *et al.*, 1985) in that antibodies which recognise different enzymes seem to recognise different compartments within cells, results obtained using such procedures are often difficult to interpret and ultimate proof requires that double labelling techniques are perfected. The compartment closest to the nucleus, which receives transport vesicles from the ER is known as the *cis* compartment and it is here that proteins are modified by mannosidase I (Kornfeld and Kornfeld, 1985: Pelham, 1989) before being moved by vesicular traffic to the medial Golgi where GlcNAc transferase I catalyses the addition of N-acetyl glucosamine to sugar chains (Dunphy *et al.*, 1985). The final compartment(s) of the Golgi complex, together with associated

tubules is(are) known as the *trans* Golgi network (TGN: Griffiths and Simons, 1986: Geuze and Morre, 1991) which contains enzymes that complete the processing of many oligosaccharide chains, for example galactosyltransferase and sialyltransferase (Roth *et al.,* 1986). The compartmentalisation and polarisation of the Golgi complex were originally suggested as a result of conventional electron microscopy observations (Farquhar and Palade, 1981) and additional evidence has since been provided by biochemical studies (e.g. differential gradient centrifugation can be used to separate enzymes associated with the organelle, suggesting that they reside in distinct membrane bound compartments) and immunocytochemistry (Goldberg and Kornfield, 1983: Dunphy and Rothman, 1983).

In addition to being the 'carbohydrate factory' of the cell, gathering substrates to be used in the modifications of oligosaccharide chains from the cytosol the Golgi complex is also the site where a number of other biosynthetic processes occur, including the formation of glycolipids, and in plants it is in this organelle that extracellular polysaccharides are produced (Mellman and Simons, 1992). It is from the TGN that proteins are sorted to their various cellular destinations (Griffiths and Simons, 1986) such as the lysosome, secretory vesicles or specific plasma membrane domains (Huttner and Tooze, 1989).

3.2. Evidence for the existence of a Golgi apparatus in yeast cells

Various modifications of the N-linked glycans that are attached to proteins in the ER of yeast cells that convert them to the form found in secreted proteins have been shown to be carried out by a membrane fraction that is distinct from the ER (Esmon *et al.*, 1981: Kukuruzinska *et al.*, 1987). The similarity of these modifications to those carried out by enzymes located in the Golgi apparatus of mammalian cells suggests that they occur in an organelle present in yeast cells that is equivalent to the Golgi complex found in mammalian cells.

Although no structure with morphology similar to the mammalian Golgi has been observed in yeast cells, temperature-sensitive mutations in either *SEC7* or *SEC14* cause cells to accumulate exaggerated structures that resemble stacked cisternae at the restrictive temperature (Esmon *et al.*, 1981: Novick *et al.,* 1981). At 37°C these cells also accumulate intracellular pools of secretory proteins with characteristic post-ER modifications of oligosaccharides.

The well characterised processing of the mating pheromone α-factor (see Section 2.2) makes it a useful tool with which to study protein transport in yeast. It is possible to detect two of the modifications that pro-α-factor undergoes as it travels through the yeast secretory pathway, namely, the addition of outer chain carbohydrate to core glycans received in the ER and its maturation to α-factor following cleavage by the Kex2 protease. Work with *sec* mutants has established that both of these events occur after the protein has left the ER and before it enters secretory vesicles. In *sec7* cells the addition of outer chain carbohydrate to pro-α-factor can be detected at 37°C, but there is a substantial reduction in the rate of maturation of the pheromone (Franzusoff and Schekman, 1989). This indicates that these reactions occur in different, distinct, cellular compartments, transport between which requires Sec7p. This conclusion is supported by the fact that intracellular vesicles containing the Kex2 protease sediment in density gradients to a position distinct from those containing mannosyltransferase I (an enzyme responsible for the addition of outer chain carbohydrate to glycoproteins) (Cunningham and Wickner, 1989). These observations have led to the belief that proteins travelling through the yeast secretory pathway encounter more than one compartment after leaving the ER and before being packaged into secretory vesicles as is the case when proteins travel through the Golgi complex of mammalian cells.

Further evidence for compartmentalisation of a yeast organelle taken to be functionally analagous to the mammalian Golgi comes from pulse-chase labelling experiments carried out using *sec7* cells. At 37°C these cells accumulate a spectrum of N-linked oligosaccharide structures (ranging from core glycosylation to nearly mature

carbohydrate) associated with the ordinarily secreted glycoprotein invertase. If the modifications leading to the various forms of the protein all occurred in a single compartment then only the mature form of invertase would be expected to accumulate intracellularly (Franzusoff and Schekman, 1989). Not only do these results support the existence of different compartments in the organelle, but they also indicate that Sec7p is required for transport of proteins between the different compartments.

3.3. Visualisation of the yeast Golgi

The absence of a structure analagous to the mammalian Golgi complex in yeast has led to the cellular distribution of proteins associated with the yeast Golgi to be investigated in order to gain some insight into the morphology of this organelle. Immunofluorescence studies indicate that Kex2p is located at multiple, discrete sites within wild type yeast cells (between 1-5 per cell) and is not concentrated at a perinuclear location as may have been expected (by analogy of the organelle in which it resides with the mammalian Golgi complex) (Redding *et al.,* 1991).

The pattern of Kex2p distribution as determined by immunofluorescence in *sec18* cells at 37°C resembles that seen for a resident ER protein. This is very different from the pattern observed in the same cells at 25°C, which is the same as that observed in wild type cells. The distribution of Kex2p in *sec1* cells (which accumulate secretory vesicles at 37°C) is as that observed in wild type cells at both 37°C and 25°C. Immunofluoresence has been used to show that secretory proteins accumulate in the bud of *sec1* cells at 37°C, and the lack of localisation of Kex2p to this area of these cells demonstrates that the protein does not enter secretory vesicles. Taken together, these obsevations (Redding *et al.*, 1991) demonstrate that ordinarily the Kex2 protein progresses from the ER but is not incorporated into secretory vesicles, a conclusion consistent with its localisation to a stage in the secretory pathway between the ER and secretory vesicles. Immunofluorescence studies reveal that Sec7p (a protein whose malfunction leads to the accumulation of exagerated Golgi like structures and

intracellular pools of secretory proteins bearing post ER modifications) has the same cellular distribution as that observed for Kex2p (Franzusoff *et al.*, 1991).

From all the above evidence it seems reasonable to refer collectively to the cellular compartments encountered by proteins travelling through the yeast secretory pathway after they leave the ER and before they are packaged into secretory vesicles as the yeast Golgi. This recently described organelle is currently receiving much research interest, including work involving the two genes, *SEC7* and *SEC14* which have Golgi-related functions (Schekman, 1992). The Kex2 protein resides in a compartment of this organelle that is encountered by proteins after they have received covalent modifications carried out in other compartments of the organelle, before they are packaged into secretory vesicles which are targetted to either the vacuole or the plasma membrane (Franzusoff and Schekman, 1989: Graham and Emr, 1991).

3.4. Protein transport assays involving the yeast Golgi

Emr and his colleagues have demonstrated that Sec18p is required at multiple stages in the yeast secretory pathway by showing that individual events in the maturation of α-factor require the protein, as indicated in Figure 1-6 (Graham and Emr, 1991). The same rationale of preloading the secretory pathway of a cell with radiolabelled protein prior to the introduction of a rapid *sec* block (described in Section 2.3) has been used to show that Sec23p is required for the addition of α-1,6 mannose to the core glycosylated form and the addition of α-1,3 linked mannose to the α-1,6 mannosylated form but not for the Kex2p dependent proteolytic processing of the α-1,3 mannosylated form or the secretion of the mature peptide from the cell. These results, taken in conjunction with the knowledge that Sec18p/NSF is required for vesicle mediated transfer of proteins between individual compartments, provide evidence that each of the events in the maturation of α-factor described above occurs in a separate membrane bound compartment.

The vacuolar protein carboxypeptidase Y (CPY) is synthesised as an inactive precursor pro-CPY which carries information that results in its localisation to the yeast vacuole where it is proteolytically processed to the mature form (mCPY). It is possible to observe the transport of this protein to its final destination in permeabilised spheroplasts prepared from cells that have been radioactively labelled under conditions that kinetically trap precursor forms of CPY bearing post translational modifications characteristic of residency in the Golgi apparatus (Vida *et al.,* 1990). Transport can be followed by monitoring the appearance of mCPY, an event which requires the action of vacuolar proteinase A (*PEP4* gene product). Transport has been shown to require ATP, cytosol and it has proved impossible to reconstitute the transport in spheroplasts prepared from certain *vps* mutants confirming that the products of *VPS15*, *33* and *34* are required for the transport of proteins to the vacuole.

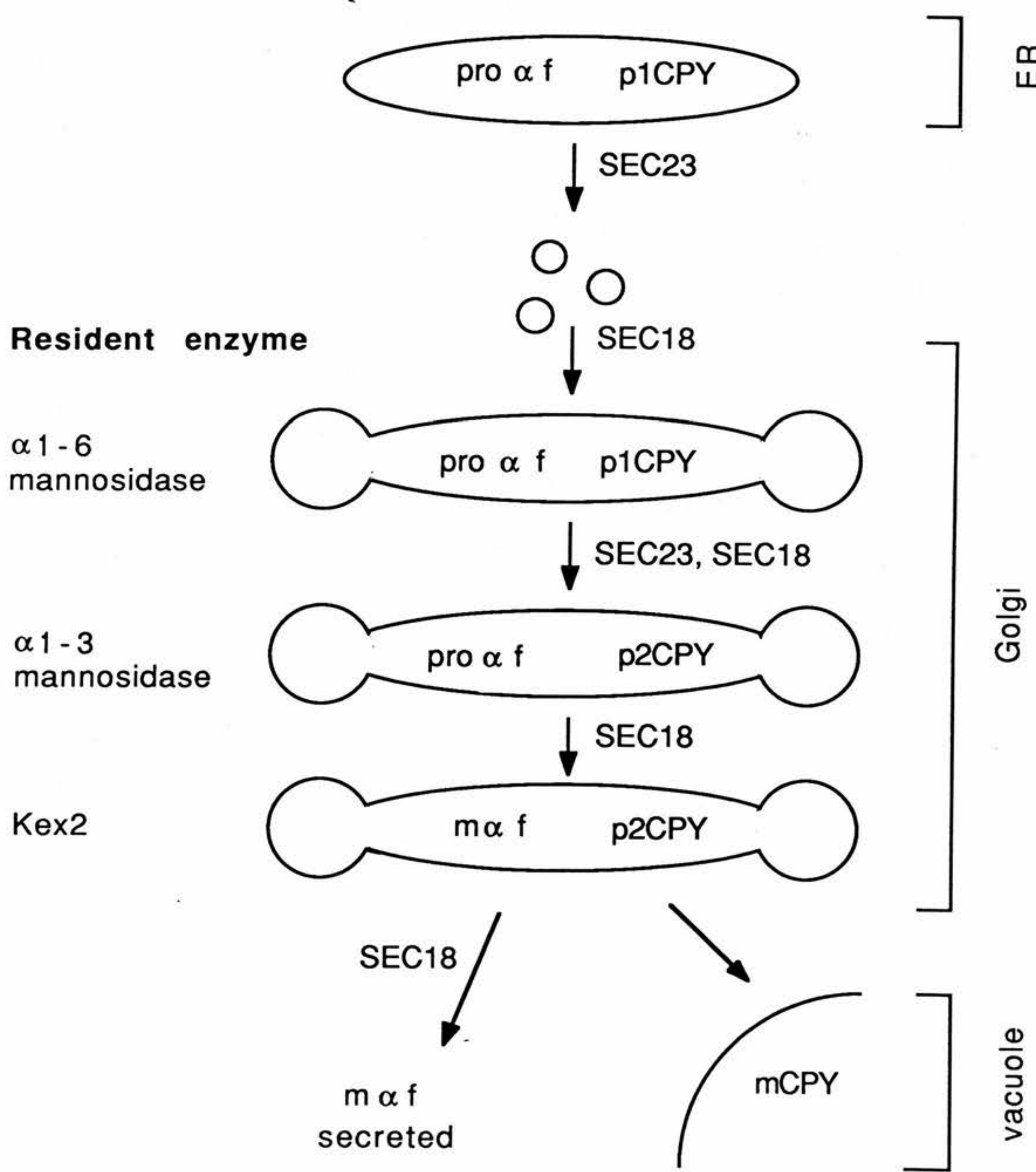
The gene encoding proCPY (*PRC1*) has been used to construct a hybrid gene which encodes a CPY-α-factor-invertase fusion protein (Graham and Emr, 1991). Cells expressing the hybrid gene secrete the invertase portion of the protein while the CPY portion remains inside the cell. From this, it is concluded that proteins are sorted to the vacuole after they have encountered the Kex2 protease which is responsible here for cleaving the fusion protein.

Taken together the above findings (Vida *et al.,* 1990: Graham and Emr, 1991) have led to a model for the compartmental organisation of post-translational modification and vacuolar protein sorting events in the yeast Golgi complex to be proposed (outlined in Figure 1-6).

Figure 1-6

A proposed compartmental organisation of the yeast Golgi

This model for the compartmental organisation of various posttranslational modification and vacuolar protein sorting events in the yeast Golgi complex has been described by Emr and his co-workers. Shown travelling through the pathway are pro-α-factor (pro α f) and the vacuolar protein CPY. The latter is depicted in the various precursor forms in which it is found as it travels through the Golgi (p1 and p2, p; precursor, m; mature). Also shown are the positions at which the gene products of *SEC18* and *SEC23* have been found to be required (adapted from Graham and Emr, 1991).



3.5. The importance of the lipid composition of organelles

SEC14 is essential for the secretory function of a late Golgi compartment in yeast and has been shown to encode a phosphatidylcholine/phosphatidylinositol (PC/PI) transfer protein (Bankaitis *et al.*, 1990). Phospholipid transfer proteins move specific phospholipids from the outer leaflet of one membrane bilayer to that of another. This finding has implicated phospholipid biosynthesis and exchange in the functioning of the secretory pathway (Cleves *et al.*, 1991a). Since *sec14* cells can function normally if they also contain mutations in genes encoding enzymes involved in PC synthesis (Cleves *et al.*, 1991b) it has been proposed that efficient transport through the Golgi complex depends on a high PI:PC ratio in the cytosolic leaflet of Golgi membranes. This proposal predicts that incubation in choline-deficient medium should rescue *sec14* mutants but this does not appear to be the case and this area remains unclear (as discussed by various authors in Trends in Cell Biology. Volume*2*, pages 69-72).

PI is a glycolipid with a large polar head group that gives the molecule an overall conical shape. PC on the other hand is zwitterionic and has a more cylindrical shape. A membrane with a high PI/PC ratio in its outer leaflet would have a very curved structure. Such a structure may be necessary for the formation of secretory vesicles from membranes. It can be seen that vesicles do bud from highly curved regions of the mammalian Golgi (i.e. the rims of Golgi saccules) which consists of otherwise planar membranes (Farquhar and Palade, 1981). Such a theory predicts that post-Golgi secretory vesicles will have a higher PI/PC ratio than their donor membrane (such a prediction will only be able to be tested following the isolation of such secretory vesicles and their donor organelle). Rather than exerting its effect through structural means, the correct phospholipid composition of membranes may be important in secretion by providing the correct environment for the functioning of proteins involved in facilitating the various stages involved in the secretory pathway. There are many examples of proteins requiring specific lipid environments to function optimally: it may be envisaged for example that the nucleation of COPs could require a high PI/PC ratio.

The exciting discovery of the function of Sec14p will undoubtedly lead to a revival of interest in the lipid composition of cellular organelles and in the lipid requirements of components involved in membrane traffic. The latter may be investigated by the incorporation of such components into liposomes containing a soluble cargo protein, delivery of which to an acceptor compartment could then be followed. Such a scheme would be able to demonstrate whether all the components involved in a protein transport step had been identified.

Section 4. Immunoisolation of subcellular organelles

4.1. Introduction

From the work and ideas discussed so far in this chapter a number of places are evident where the ability to separate one organelle from all other cellular components would be advantageous, section 3.5 for example. This would allow interpretation of results from transport assays to be made with less confusion as implied by the quote 'don't waste clean thinking on dirty enzymes' (Efraim Racker, quoted in Kornberg, 1990). The technique of immunoisolation allows this to be achieved. An antibody that has been covalently bound to a solid support (to form an immunoadsorbent or ImAd) can be used to isolate its antigen and any associated structures from a cell lysate or other biological preparation. Direct immunoisolation involves the addition of a preformed ImAd to the preparation from which the desired cellular component is to be isolated. During incubation in the preparation under appropriate conditions the ImAd will bind the antigen against which its antibody was raised. The ImAd can then be recovered, either by centrifugation, if for example agarose beads were chosen as the solid support, or by magnetism if magnetic beads had been used. Such quick and easy recovery procedures allow the 'ImAd:antigen and associated structures' complex to be washed free of any material which may bind to the complex non-specifically. Indirect immunoisolation involves the addition of free antibody to a system, after which a

matrix that will bind the antibody (as part of an antigen:antibody complex) is added (see Figure 1-7).

4.2. Immunoisolation of membranes

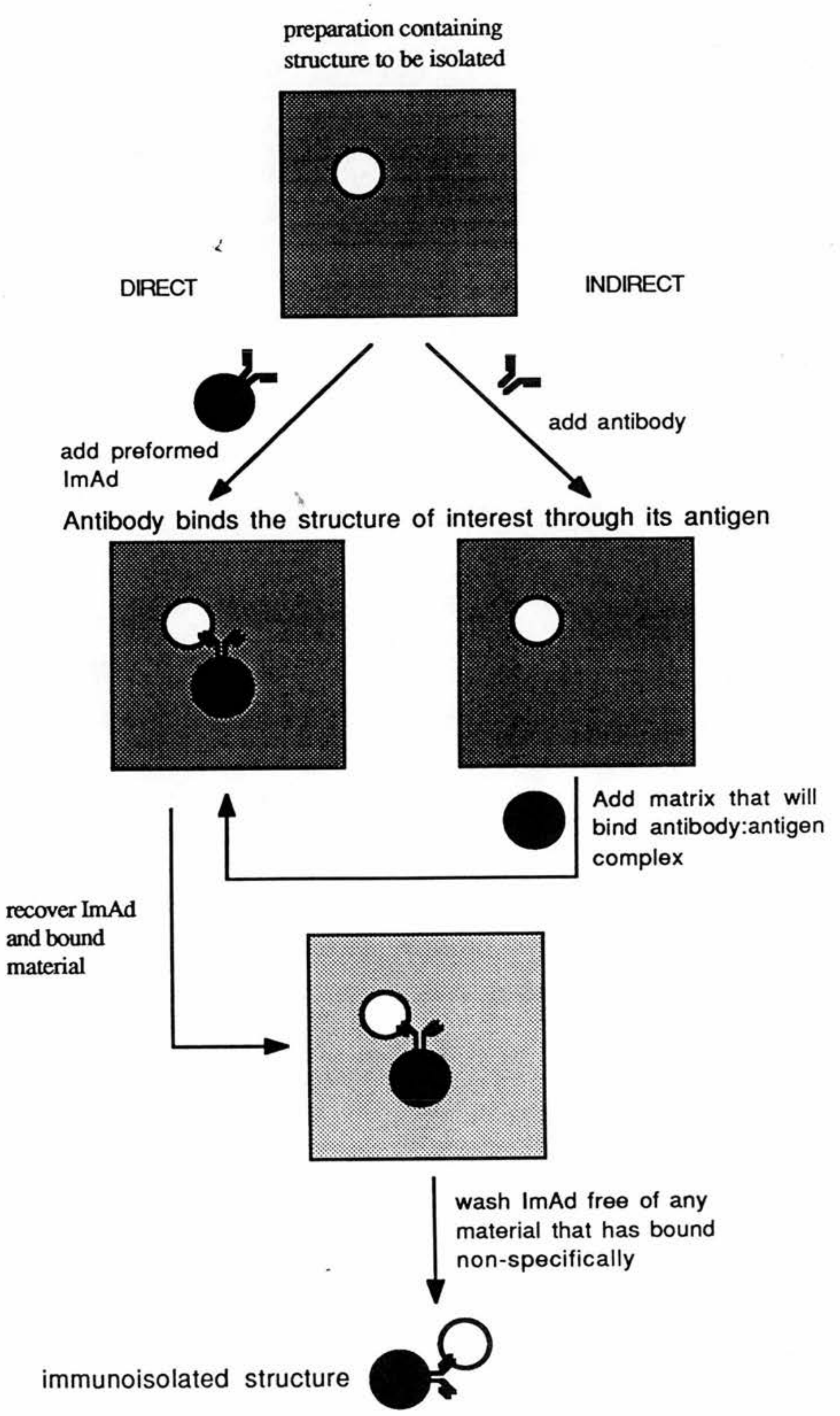
Equilibrium density gradient centrifugation is often used to separate membranes that are derived from different organelles, with the various fractions being identified by assaying for the activities of various marker enzymes. This technique has been used to prepare fractions enriched in various organelles which have subsequently been used as donor or acceptor fractions in protein transport assays (e.g. Balch *et al.*, 1984). The Golgi fraction used by Rothman and co-workers in their intra-Golgi transport assay is reported to have been between 30 and 40% pure (Balch *et al.*, 1984). The use of less contaminated fractions in such a system would offer many advantages in analysing results and although it is likely that a purer preparation could be obtained using standard techniques this is likely to be more time consuming than the preparation of such a fraction by immunoisolation. Immunoisolation takes advantage of an antigen's localisation to a particular cellular structure in order to purify that structure by using the antigen as a handle. Since it relies on the specific interaction between an antibody and its antigen this technique offers advantages in the purification of membrane fractions over more traditional methods of isolating such fractions which rely on physical differences between membranes in that membranes of a higher purity may be obtained in a shorter period of time.

When membranes are required to study protein transport, immunoisolation offers an advantage over equilibrium density gradient centrifugation in that it facilitates recovery of the membrane fraction after the transport assay has been carried out. This may be useful as it allows the analysis of the fraction before and after the reaction and can be used to study any changes which may have occured during the reaction.

Figure 1-7

Immunoisolation of cellular structures

A diagramatic representation of the theory behind the technique of immunoisolation is shown here comparing direct and indirect forms of the technique.



The technique also allows the 'sidedness' of membrane vesicles to be chosen. This can be achieved using an antibody that specifically recognises its antigen through a domain known to be located on a particular side of a membrane (Figure 1-8).

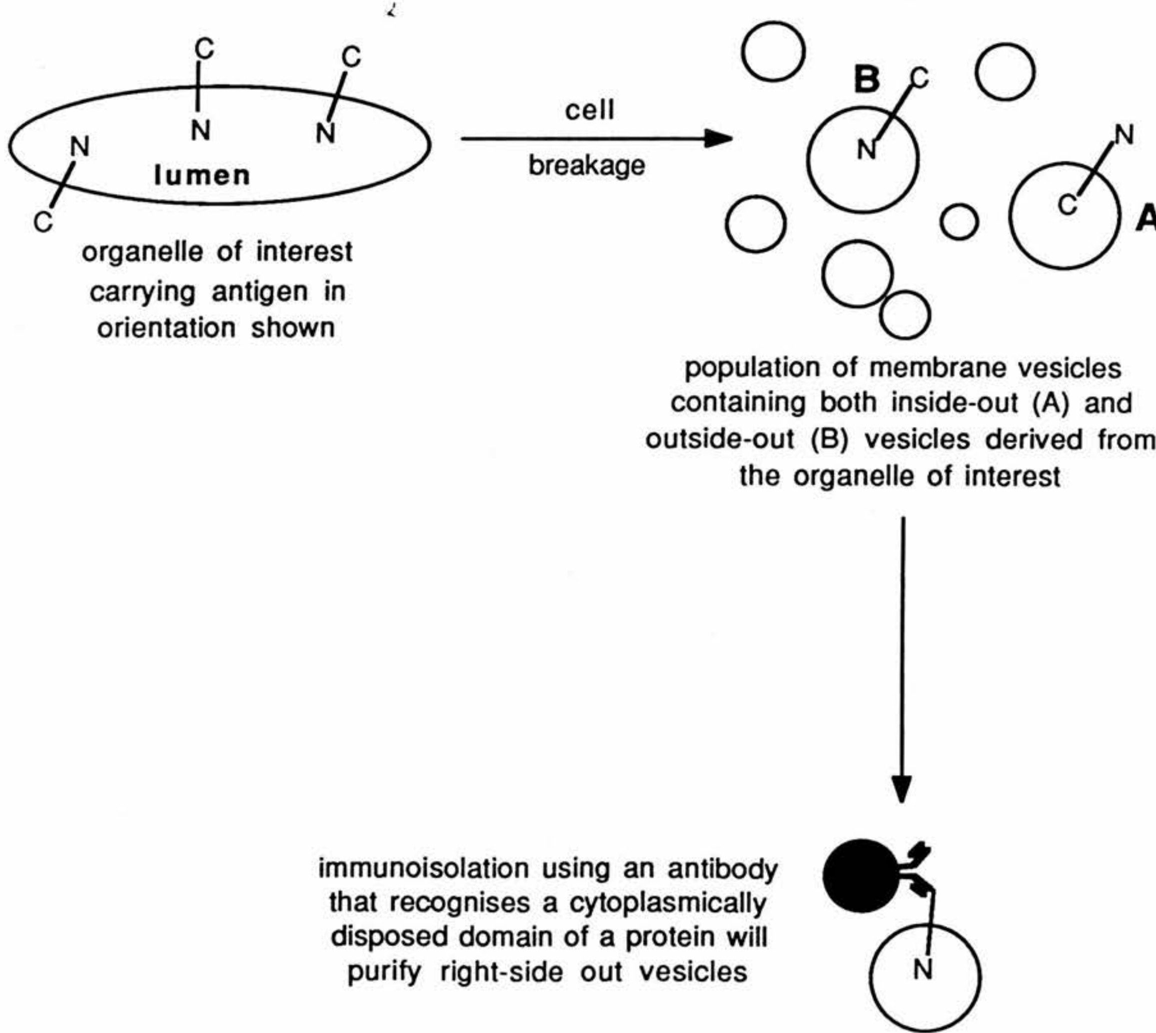
The plasma membrane of epithelial cells is divided into two distinct domains, with different protein compositions. Transport vesicles that deliver proteins from the TGN of such cells to the plasma membrane are targetted either to the apical domain or the basolateral domain. VSV-G-protein is targetted to the basolateral plasma membrane of MDCK cells, whereas a membrane protein of influenza virus, haemagglutin A is targetted to the apical domain of the same cells. Antibodies raised against cytoplasmic domains of the two virally-encoded proteins have been used to demonstrate that these two proteins are packaged into distinct sets of transport vesicles in cells infected with the two viruses (Wandinger *et al.*, 1990). The two sets of vesicles, immunoisolated using the antibodies immobilised on agarose beads from a gradient purified membrane fraction prepared from virally infected cells, both contain a subset of proteins present in the fraction from which they were isolated. Some of these proteins are present in both types of vesicles and are believed to be involved in budding and/or fusion events, whereas proteins that are unique to one type of vesicle may be involved in specific recognition events such as protein sorting or vesicle targetting.

Antibodies that recognise the cytoplasmic domain of G-protein have also been used to immunoisolate endocytotic intermediates from extracts prepared from cells whose endocytotic pathways had been allowed to proceed for various lengths of time (Gruenberg *et al.*, 1989). Both of the examples outlined above allowed the characterisation of the isolated intermediates.

Figure 1-8

Immunoisolation of membrane vesicles

This figure demonstrates how an antibody which recognises a specific domain of a membrane protein whose orientation is known may be used to isolate vesicles of known sidedness derived from the membrane in which the antigen resides.



Vesicles involved in protein transport are often present in cells in very small amounts, and are short lived intermediates. Incubation of *sec* mutants at their restrictive temperature can increase the cellular population of functional transport vesicles, and this fact has been taken advantage of in the purification of post-Golgi secretory vesicles from yeast from blocked *sec6* cells (Walworth and Novick, 1987). This purification, involving gel filtration chromatography as well as various forms of centrifugation, allowed the characterisation of these vesicles and led to the identification of two membrane proteins associated with them. It may be possible to use this information to develop a procedure which would allow these same vesicles to be immunoisolated. Such a procedure would offer an advantage over the purification procedure described, in that it would allow the vesicles to be added to, and then later removed from cell free systems with ease, allowing their function to be studied.

4.3. Immunoisolation in conjunction with protein transport assays

SEC7 encodes a phosphoprotein with a molecular weight of 230K (Achstetter *et al.*, 1988) that is required at a number of stages in the secretory pathway (Franzusoff and Schekman, 1989). Sec7p is present in wild type yeast lysates in approximately equal proportions of soluble and membrane associated forms. Antibodies raised against the protein have the ability to block the formation of the highly glycosylated form of pro-α-factor in the transport assay described in Section 2.6 (Franzusoff *et al.*, 1992). The addition of the antibodies did not affect the budding of vesicles from the ER, since the core glycosylated form of the protein still appeared in the medium speed supernatant (MSS-that does not contain the ER) in their presence, but this did not receive outer chain glycosylation indicating that delivery to the Golgi had been blocked. The transport assay has been used to show that the Golgi present in a MSS treated with the Sec7p antibodies is competent as an acceptor compartment, but the transport vesicles containing core-glycosylated pro-α-factor are incompetent for fusion. Protein A-Sepharose added to such a MSS results in the recovery of membrane vesicles devoid of marker enzymes characteristic of the ER (Franzusoff *et al.*, 1992). Examination of this

immunoisolated fraction by electron microscopy reveals that it contains vesicles similar in size to those accumulated by class II ER-accumulating *sec* mutants. This technique has opened a way to the characterisation of these vesicles, although since Sec7p has been shown to be required at multiple stages in the yeast secretory pathway the preparation may contain other vesicles apart from those derived from the ER and this must be taken into consideration. Initial characterisation studies (from elecron microscopy) have been taken to suggest that Sec7p may be involved in the formation of a coat around these transport vesicles a theory which fits with its involvement in multiple stages of the secretory pathway.

Section 5. Ouline of the project

The aim of this project was to investigate the use of immunoisolation procedures for the purification of specific membrane fractions from yeast. More specifically, a polyclonal antiserum was to be raised against the cytoplasmically disposed C-terminal domain of the Kex2 protease which could then be used to isolate membranes derived from the compartment of the yeast Golgi containing this protein. The isolation of such membranes would allow the characterisation of the Kex2p compartment with respect to its structure, by examining the composition of immunoisolated material, and also its function, by developing transport assays in which it may be used.

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Chapter 2

Materials and methods

Materials

2.1. Chemicals, enzymes and antibodies.

All chemicals were obtained from Sigma Chemical Co. or BDH Chemicals. Trans-label [^{35}S]-met (1136 Ci/mmol) was from ICN Radiochemicals. DNA modification enzymes were from BRL. Taq polymerase was from Promega limited. Zymolyase 100T was from the Seikagaku Kogyo Co. Japan, lysozyme was from Sigma Chemical Co. Antibodies not produced during the course of this project were obtained from the Scottish Antibody Production Unit (unless otherwise stated), the anti-ampicillinase antibodies were from 5 prime-3 prime Inc. Enhanced chemiluminescence (ECL) kit and Hyperfilm-MP were from Amersham International. Pansorbin (*Staphylococcus aureus* cells, standardised) was from Calbiochem. The synthetic substrate for the Kex2 assay (b-QRR MCA) was from the Peptide Institute Inc., Japan. Nitrocefin was from BBL Microbiology Systems. IgG-Sepharose (Fast Flow) was from Pharmacia LKB. Affi-gel 10 was from Bio-Rad Laboratories, as were the Poly Prep chromatography columns. Media components were from Difco Laboratories.

2.2. Bacterial and yeast strains

The strains of *E. coli* and *S. cerevisiae* used in this study are listed in Table 1 (see Appendix). Derivatives of these are not listed, but are described in appropriate text. Transformants are denoted by listing the strain, followed by the plasmid with which it has been transformed in parenthesis.

2.3. Media

Bacterial cultures were grown in complete medium (Luria broth; L-broth) containing 1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract and 0.5% (w/v) NaCl. Antibiotics were added to this as required. Bacto agar was added to the above to 1.5% (w/v) when solid medium was required.

Yeast cultures were grown in either rich medium (YPD; containing 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone and 2% glucose) or minimal (selective) medium (SD; containing 0.67% (w/v) Bacto yeast nitrogen base without amino acids and 2% (w/v) glucose) to which the following were added, to the following final concentrations, as required; histidine (20µg/ml), leucine (30µg/ml), tryptophan (20µg/ml), uracil (20µg/ml). Where stated, the carbon source supplied in the medium was galactose which was added to the above in place of glucose to make (YPG and SG). 2% (w/v) Bacto agar was added, to 2% (w/v), to the above when solid medium was required.

Sporulation media consisted of 0.1% (w/v) Bacto yeast extract, 1% potassium acetate and 0.05% (w/v) glucose.

2.4. Plasmids

All plasmid DNA that was used in this study is described in Table 2 (see Appendix).

2.5. DNA manipulations

All general DNA manipulation techniques, including restriction endonuclease cleavage, extraction with phenol, precipitation in ethanol, ligation and PCR were performed as described by Maniatis *et al* (1982) and Sambrook (1989).

Gel electrophoresis, for the separation and visualisation of DNA fragments, was routinely carried out using agarose gels of between 0.6 and 1.2% (w/v) agarose; the size of fragments of DNA was estimated by comparing their mobility through an agarose gel with that of fragments of known size liberated by digestion of phage λ DNA with either *Pst*I or *Bst*EII.

2.6. Transformation of bacterial and yeast cells

DNA was transformed into bacterial cells which had been treated with $CaCl_2$ as described by Maniatis *et al* (1982). Yeast cells were made competent for transformation by using LiOAc as described by Ito *et al.* (1983).

2.7. Preparation of yeast genomic DNA

A 5ml of culture of yeast cells was grown overnight in YPD. Cells were harvested from this and resuspended in 0.5ml 1M sorbitol, 0.1M EDTA (pH7.5). Zymolyase was added to 20µg/ml and the cells were incubated at 37°C for 1 hour. Spheroplasts were harvested by centrifugation for 2 mins at 4000g and resuspended in 50mM Tris.HCl (pH7.4), 20mM EDTA. 50µl 10% SDS was added prior to incubation at 65°C for 30 mins, after which 200µl 5M potassium acetate was added. The sample was incubated on ice for 1 hour and then centrifuged at 10 000g for 5 mins. An equal volume of isopropanol was added to the resulting supernatant, and after 5 mins

incubation at room temperature the sample was centrifuged for 10 mins at 10 000g. The pellet thus obtained was air-dried and then resuspended in 300µl TE buffer (10mM Tris.HCl (pH8.0), 1mM EDTA). 30µl of 3M NaOAc (pH5.2) and 200µl isopropanol were added and the sample was centrifuged for 5 mins at 10 000g. The final pellet was resuspended in 200µl TE buffer.

2.8. Determination of yeast mating type

To establish whether a yeast strain was *MATa* or *MATα*, cells were mixed separately with the strains DC14 and DC17 under conditions that select for the formation of prototrophic diploids. *MATα* strains formed such diploids with DC14 and *MATa* strains with DC17.

2.9. Electrophoretic separation of proteins

Electrophoretic separation of proteins was performed using SDS polyacrylamide gels following the basic procedures described by Laemmli (1970) using the various solutions detailed below.

Separating gel buffer:	0.75M Tris.HCl (pH8.8), 0.2% (w/v) SDS
Stacking gel buffer:	0.25M Tris.HCl (pH6.8), 0.2% (w/v) SDS
Acrylamide solution:	44% (w/v) acrylamide, 0.8% (w/v) N;N'-methylene-bisacrylamide
Electrophoresis buffer:	0.125M Tris. 0.2M glycine, 0.1% (w/v) SDS (gives pH8.3 without adjustment)
SDS sample buffer:	0.0625M Tris.HCl(pH6.8), 20% (w/v) glycerol, 4% (w/v) SDS, 5% (w/v) β-mercaptoethanol

Routinely, either a 12% (w/v), 10% (w/v) or a 6% (w/v) separating gel, with a 5% (w/v) stacking gel, was used to achieve separation of proteins.

Following electrophoretic separation, protein bands were visualised by staining with Coomassie blue: the gel was allowed to equilibrated with a solution of 0.25% (w/v) Coomassie Brilliant Blue dissolved in 50% (v/v) methanol, 7% (v/v) acetic acid. Destaining of the gel was achieved by gently agitating the gel, immersed in 10% (v/v) methanol, 7% acetic acid.

2.10. Preparative SDS-PAGE and electroelution

The sample from which the desired protein was to be purified was subjected to electrophoretic separation using an appropriate polyacrylamide gel with a single well (capable of holding 1ml of sample) across its top. Following staining of the gel with Coomassie blue a slice of the gel containing the protein of interest was excised. The protein was extracted from this gel slice by dividing it into small fragments (approx 2mm x 2mm) and placing them into a length of dialysis tubing containg 5ml electrophoresis buffer. Electroelution was achieved by placing the sealed dialysis tubing containing the gel in an electrophoresis tank containing electrophoresis buffer and applying a voltage of 125V for 5 hours. Following this, the buffer into which the protein had been eluted was dialysed, at 4^oC, against distilled water.

2.11. Fluorography

Following electrophoretic separation of radiolabelled proteins on an appropriate polyacrylamide gel the gel was incubated on a shaking platform in 25% methanol, 7% acetic acid for 1 hour. The gel was then washed (4 x 10 mins) in distilled water, before being incubated in 1M sodium salicylate for 1 hour. The gel was washed as before, prior to being vacuum dried onto blotting paper. X-ray film was exposed to the dried gel at -70^oC.

2.12. Transfer of proteins onto nitrocellulose

2.12a. Semi-dry electroblotting

Where required, proteins that had been separated on polyacrylamide gels were transferred onto nitrocellulose by a semi dry blotting procedure (using an LKB semi dry blotting apparatus). Six pieces of 3MM paper and one piece of nitrocellulose were cut to the same size as the gel to be blotted. Two of the pieces of 3MM paper were soaked in anode buffer 1 (0.3M Tris.HCl (pH10.4), 20% (v/v) methanol, 0.1% SDS) and placed, one on top of the other, on the anode plate. A piece of 3MM paper soaked in anode buffer 2 (25mM Tris.HCl (pH10.4), 20% (v/v) methanol, 0.1% (w/v) SDS) was placed on top of these, followed by the nitrocellulose which had been soaked in water and then the gel which had been soaked in cathode buffer (25mM Tris.HCl (pH9.4), 20% (v/v) methanol, 0.1% (w/v) SDS, 40mM 6-amino-*n*-hexanoic acid). The remaining 3 peices of 3MM paper were soaked in cathode buffer and layered on top of the gel. The cathode plate was placed on top of the stack and transfer was achieved by applying a current of 0.8mA per cm^2 gel area.

2.12b. Wet electroblotting

For the detection of Ste2p on immunoblots it was necessary that the buffers used did not contain methanol. When this protein was to be detected the proteins were transferred using a wet blotting apparatus. The SDS-polyacrylamide gel containing the separated proteins was placed next to a sheet of nitrocellulose that had been cut to the same size as the gel and was then sandwiched between two sets of three sheets of 3MM paper that had been soaked in blotting buffer (125mM Tris.HCl (pH7.4), 200mM glycine). Transfer was achieved by placing this sandwich in a tank containing blotting buffer and a current of 0.8 amps was applied for 2 hours (with the nitrocellulose closest to the anode).

2.12c. Dot blotting assay: screening culture supernatants (for monoclonal antibody production)

Yeast lysate (diluted to 0.2mg/ml and solubilised with 0.05% Triton X-100) was applied to a sheet of nitrocellulose (soaked in distilled water) using a Biorad Bio-Dot microfiltration apparatus by passive filtration. Unfilled sites on the nitrocellulose were blocked with 0.5% Tween-20 in TBS (1 hour). The nitrocellulose membrane was put back in the dot-blotting apparatus with the positions of the dots corresponding to the wells. The apparatus was sealed and various culture supernatants were added to the wells. Following overnight incubation, the filter was processed as described for immunoblot analysis (2.13.), using anti-mouse-IgG and anti-mouse-IgM antibodies.

2.12d. Ponceau S staining

The presence of proteins on nitrocellulose filters was detected using Ponceau-S. The nitrocellulose was immersed in 0.2% (w/v) Ponceau-S in 3% (w/v) TCA. Destaining was achieved by washing the nitrocellulose with distilled water and the staining was reversible upon washing the filter with TBS (10mM Tris.HCl (pH7.4) 1.5M NaCl).

2.13. Immunoblot analysis

Unfilled sites on a nitrocellulose filter onto which proteins had been transferred were filled by gently agitating the filter in blocking buffer (5% (w/v) non-fat dried milk, 1% Tween-20 in TBS; 10mM Tris.HCl (pH7.4) 150mM NaCl) for 1 hour. The filter was then exposed to primary antibody for one hour with gentle agitation. Antibody preparations were presented to the filter, diluted in blocking buffer as described in the text.

Following exposure to primary antibody , the filter was washed, once for 15 mins and then 3 times for 5 mins with washing buffer (1% (v/v) Tween-20 in TBS). Where required, the filter was then exposed to secondary antibody (routinely, a donkey anti-rabbit IgG-HRP conjugate used at a dilution of 1 in 5000 in blocking buffer for the

detection of rabbit antibodies; and either an anti-mouse-IgG (whole molecule) or an anti-mouse-IgM (μ chain specific) antibody for the detection of mouse antibodies) for 20 mins. For the detection of protein A fusion proteins only one antibody (a rabbit anti-bovine IgG-HRP conjugate was used at a dilution of 1 in 10 000 in blocking buffer) was required, and the filter was exposed to this for 20 mins. Prior to the detection of the HRP-conjugated antibody, the filter was washed as before, and then once briefly with TBS.

Two different methods of detecting HRP conjugated antibodies on immunoblots were used in this study. Immunoblots presented in the first part of Chapter 3 (up to and including Figure 3-7) were developed using 4-chloro-1-naphthol. This was achieved by placing the filter in developing solution (containing 20ml of a solution of 4-chloro-1-naphthol (3mg/ml in methanol) and 20ml of a 30% (w/v) H_2O_2 solution per 100ml) and watching for the appearance of a dark brown precipitate indicating the presence of HRP on the filter. The reaction was stopped by washing the filter liberally with distilled water and then storing in TBS in the absence of light. It was important that immunoblots developed in this way were photographed as soon as possible as the colour tended to fade fairly rapidly.

Immunoblots presented later in this study were developed using enhanced chemiluminesence (ECL) with ECL reagent in accordance with the manufacturers instructions.

N.B. In order to include molecular weight marker proteins on immunoblots, the nitrocellulose filter was stained with Ponceau S prior to immunoblot anlysis. This revealed the positions of marker proteins which had been separated on the gel, these positions were then marked on the filter using indelible ink.

2.14. Production of hybrid proteins from *E. coli*

2.14a. Induction of expression from the *lacZ* promoter

A culture of bacteria harbouring a plasmid containing a gene under the control of the *lac Z* promoter (e.g. pKpra) was grown in selective medium to an OD_{600} of 0.8. Expression was induced by the addition of IPTG to a final concentration of 0.25mM and the culture was left growing at 37°C for a further 4 hours.

2.14b. Induction of expression from the P_R promoter

A culture of bacteria harbouring a plasmid containing a gene under the control of the P_R promoter (e.g. a pEX vector) was grown at 30°C in selective medium to an OD_{600} of 0.4. Expression was induced by incubating the culture at 42°C for 4 hours.

2.14c. Fractionation of a bacterial culture by differential centrifugation (Preparation of inclusion bodies)

Cells were harvested by centrifugation (5 mins at 5000rpm in a Beckman JA-20/JA-14 rotor), washed once with, and then resuspended in, one twentieth of the culture volume 100mM Tris.HCl (pH7.4). Lysozyme was added to a final concentration of 1mg/ml and the cells were left on ice for 20 min. Cell lysis was achieved using sonication (5 bursts of 30 seconds with 2 min cooling intervals on ice). The lysate was clarified by centrifugation in a Beckman JA-20 rotor (3000rpm for 5 mins), to yield the pellet P3. The resultant supernatant was subjected to further centrifugation in the same rotor (20 mins at 20 000rpm) to yield the soluble S20 and the insoluble P20 fractions. For comparison of the protein composition of the various fractions, both the P3 and the P20 were resuspended in one twentieth of the original culture volume 100mM Tris.HCl (pH7.4) before subjection to electrophoretic separation.

2.14d. Use of IgG-Sepharose in the purification of Spa-fusion proteins

A soluble fraction (of volume V) was prepared from an appropriate bacterial culture (Chapter 3) and was incubated overnight at 4°C on a rotating wheel to allow gentle

mixing with IgG-Sepharose (V/10) which had been equilibrated, by washing, with TST buffer (50mM Tris.HCl (pH7.4), 150mM NaCl, 0.05% Tween-20). The IgG-Sepharose was washed eight times with TST buffer before being resuspended in elution buffer (V/5) (0.5M acetic acid adjusted to pH3.4 with ammonium acetate). Elution of material bound to the IgG-Sepharose was achieved by gentle mixing at 4°C for 20 mins. A second elution was performed for a further 10 mins and the two eluates were pooled and dialysed against distilled water.

2.15. Production of polyclonal antibodies from rabbits

2.15a. Immunisation of rabbits and collection of serum

For immunisation with spa fusion proteins, 100µg of the protein (in a 250µl volume) was emulsified with an equal volume of Freund's complete adjuvant. This material was administered to young New Zealand white rabbits by subcutaneous injection. Six weeks after the initial injection a subcutaneous booster of the same amount of the protein was administered in Freund's incomplete adjuvant. A second boost was administered in this way six weeks after the first, 10 days after which, 5ml of blood was collected from the animal. Serum was prepared from this blood sample by allowing it to stand at room temperature for 1 hour and then at 4°C for 24 hours. The serum was separated from clotted material by centrifugation (10 mins at 10 000rpm in a Beckman JA-20 rotor) and was passed through a 0.22µm Millipore filter. After it had been ascertained that the serum contained the desired antibodies (e.g.by checking for recognition of the appropriate βgal fusion protein) the animal was sacrificed (provided that it was less than 14 days after the last boost) and serum was prepared from the blood obtained. Serum was stored in 1ml aliquots at -20°C.

2.15b. The use of βgal fusion proteins to affinity purify antibodies

2ml (slurry volume) Affi-gel 10 was washed once with 10ml water and once with 10ml 0.1M Hepes (pH7.4), the Affi-gel was allowed to settle out by gravity, on ice, in

between these washes. Routinely, 500μg of the appropriate fusion protein, resuspended in 10ml 0.1M Hepes (pH7.4) was bound to the matrix by allowing gentle mixing at $4^{o}C$ for 2 hours or overnight. The affinity matrix was washed using 0.1M Hepes (pH7.4) until the A_{280} of the buffer after washing was zero. Any remaining reactive groups on the Affi-gel were quenched by gentle mixing with 10ml 0.2M glycine HCl (pH8.0) for an hour at $4^{o}C$. Following two washes with 0.1M Hepes (pH7.4), the affinity matrix was gently mixed with elution buffer (0.2M glycine HCl (pH2.8) for 5 min at room temperature. After restoration to pH7.4 using the 0.1M Hepes buffer, the affinity matrix was incubated at $4^{o}C$ on a rotating wheel for 2 hours or overnight with 5ml immune serum (raised against the appropriate protein) before being packed into a 10ml Poly Prep chromatography column. The column was washed with 0.1M Hepes (pH7.4) until the buffer coming through the column had an $A_{280} = 0$ (approx. 5 bed volumes). Elution of antibodies bound specifically to the column was achieved using 0.2M glycine HCl pH2.8. Fractions (1ml) were collected into tubes containing 75μl 1M Tris.HCl (pH8.0) (to give a final pH 7.4) with A_{280} being monitored. Using this procedure, generally it was the first 5 fractions eluted from the column which were pooled giving a 5ml sample with an $A_{280} = 0.05$. This was divided into aliquots (100μl) and stored at $-20^{o}C$ following the addition of sodium azide to 0.1%.

2.16. Production of antibodies from mice

A six week old mouse was immunised subcutaneously with 100μg Spa-Pma1p fusion protein (Chapter 3). Three weeks later it was immunised with a similar dose of the same antigen intraperitoneally, a procedure that was repeated once more three weeks later again. Three days after the final immunisation the mouse was bled to death. 2ml sera was prepared from the mouse in the same way as described for the preparation of rabbit sera. The mouse's spleen was removed under sterile conditions and was used to prepare hybridoma cell lines by Dr J. Haywood (as described in Tugal, 1991).

The supernatants from these cell lines were tested for reaction with the Pma1p in yeast cell lysates during the course of this study.

2.17. Preparation of yeast cell lysate/homogenate

Where a lysate was required for the preparation of membrane vesicles all cell breakages were achieved in the presence of 0.8M sorbitol.

2.17a. Yeast cell glass bead lysate

Cells were harvested (by centrifugation for 5 mins at 5000rpm in a Beckman JA-20/JA-14 rotor) from yeast cultures that had been grown to an OD_{600} of 0.7-1.0, washed once in distilled water and resuspended to 100 OD units/ml in lysis buffer (200mM Tris.HCl (pH7.4), 0.7M sorbitol, 2mM EDTA.) containing protease inhibitors (1mM PMSF, 0.4mg/ml each of pepstatin, leupeptin, chymostatin and antipain, 1mM EDTA, 1mM EGTA.). Glass beads were added to the level of the meniscus and the tubes were vortexed 4 x 30 seconds (with two minute cooling intervals on ice). The cell lysate was collected by centrifugation, through the glass beads, at 3000g for 10 mins. This step resulted in a pellet of unlysed cells and cell debris and a supernatant of cell lysate.

2.17b. Yeast cell homogenate

Cells were harvested as above, washed and then resuspended in spheroplast buffer (1.4M sorbitol, 50mM potassium phosphate (pH7.4), 10mM NaN_3, 40mM β-mercaptoethanol) containing zymolyase (5mg/ml). Cell wall digestion was achieved at 25°C, and this incubation was carried out until at least 80% of the cells present had been converted to spheroplasts (normally 30 mins; see 2.17c below). Spheroplasts were harvested (2 mins at 4000g), washed once in spheroplast buffer before being homogenised (7 strokes) on ice in 1ml/100 OD units of the original culture homogenisation buffer (0.8M sorbitol, 10mM triethanolamine, 1mM EDTA, brought to pH7.4 with acetic acid) containing protease inhibitors (as above). The homogenate was cleared of cell debris by centrifugation (2 mins at 4000g) before use.

2.17c. Spheroplast assay

To estimate the proportion of cells in a sample that had had their cell wall digested 10µl of the sample was diluted to 1ml in distilled water and 10µl to 1ml in spheroplast buffer. The OD_{600} of the two diluted samples was recorded and an index of the proportion of cells in the sample that had been converted to spheroplasts was taken as;

$$\frac{OD_{600} \text{ in spheroplast buffer} - OD_{600} \text{ in distilled water}}{OD_{600} \text{ in spheroplast buffer}} \times 100$$

2.18. Fractionation of yeast cell extracts by differential centrifugation

Yeast cell lysates/homogenates were fractionated using differential centrifugation by subjecting them to a one hour centrifugation at 10 0000g to yield a high speed supernatant (S100) and a membrane pellet (P100). This was performed using a Beckman TL100-3 rotor (55 000rpm for 1 hour).

2.19. Radiolabelling of yeast proteins

A 100ml culture of the yeast strain to be radiolabelled was grown in selective medium to an $0D_{600}$ of approximately 0.5. A 10ml aliquot of this was removed and had 10µCi of ^{35}S-methionine added to it. Both the 10ml aliquot, and the remaining 90ml culture were allowed to grow for a further 1 hour. After this time, the 10ml aliquot was returned to the culture, which was harvested for subsequent analysis as desired.

2.20. Immunoisolation techniques

2.20a. Preparation of Kex2-Immunoadsorbent (Kex2-ImAd)

All steps described here were carried out at 4°C. Following all washes and incubations the cells were pelleted by centrifugation for 2 mins at 4000g. After an initial wash in binding buffer (20mM Hepes pH7.2, 2mM $MgCl_2$, 150mM KCl) standardised Pansorbin cells were resuspended in blocking buffer (binding buffer containing 10mg/ml bovine serum albumin) using 1ml/50µl original 10% (w/v) cell suspension volume used and placed on a rotating wheel for 1 hour. The blocked cells were washed for 15 mins in binding buffer before the addition of affinity-purified anti-Kex2C antibody. For the production of the standard ImAd which was used throughout this study, 1µl of affinity purified antibody (5µg protein) was added for each µl of cell suspension used and the sample volume was made up to 500µl with binding buffer. After incubation on a rotating wheel for 3 hours or overnight the ImAd was washed once (15 minutes) in binding buffer and once with lysis buffer or homoginisation buffer, as appropriate, prior to use.

2.20b. Recovery of a Kex2-ImAd bound fraction from yeast

Kex2-ImAd was reususpended using, routinely 300µl (approximately 300µg protein) freshly prepared yeast cell lysate (prepared using either glass bead lysis or homogenisation) and was left to incubate on a rotating wheel at for 3 hours to allow gentle mixing. The ImAd was retrieved from these samples by centrifugation (4000g for 2 mins) and was washed 3 times (twice by simple resuspension and 1 x 15 mins on the rotating wheel) with lysis buffer or homogenisation buffer as appropriate (all steps described here were carried out at 4°C).

2.20c. Recovery of an IgG-Sepharose bound fraction from yeast

The procedure described for the preparation of a Kex2-ImAd bound fraction from yeast was followed using 50µl (slurry volume) IgG-Sepharose in place of Kex2-ImAd.

2.21. Enzyme assays

2.21a. Kex2 activity assay

The sample being assayed for Kex2 protease activity was added to 50µl Kex2 assay mix (200mM Hepes (pH7.0), 1mM $CaCl_2$, 0.5mM PMSF, 0.1mM L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone, 1% (w/v) Triton-X-100, 100mM *t*-butoxycarbonyl-Gln-Arg-Arg-4-methylcoumarin-7-amide (bQRR-MCA)). Reaction mixtures were incubated at 37°C for 30 mins after which time the reaction was terminated by the addition of 0.9ml 125mM $ZnSO_4$ and 0.1ml of a saturated $Ba(OH)_2$ solution. The precipitate formed upon the addition of the $Ba(OH)_2$ was removed by centrifugation in a microcentrifuge for 1 min. The amount of Kex2 protease activity in the sample was determined by detecting free 7-amino-4-methylcoumarin (AMC) released from bQRR-MCA following cleavage by Kex2 (after the pair of arginine residues). The amount of AMC in the sample at the end of the assay was determined fluorimetrically, λ(excitation) = 385nm, λ(emission) = 465nm. Assay results are expressed in arbitary units of fluorescence.

2.21b. β-lactamase (ampicillinase) activity

5mg nitrocefin was dissolved in 0.5ml DMSO before being diluted to 0.5mg/ml with 0.1M sodium phosphate (pH7.0). 50µl of this stock nitrocefin solution was added to 900µl 0.1M sodium phosphate (pH7.0) in a cuvette. The sample to be assayed was added to this cuvette in a 50µl volume and the increase in A_{490} was followed.

2.21c. Carboxypeptidase Y assay

50µl of the sample to be assayed was added to 1ml of assay mix containing, 0.125mg/ml L-amino acid oxidase, 0.2mg/ml peroxidase, 0.5mM $MnCl_2$, 0.1mg/ml dianisidine.HCl and 5mM peptide substrate (*N*-carbobenzoxy-L-phe-L-leu) in 0.1M potassium phosphate buffer (pH7.0). The complete mixture was incubated at 37°C for 90 mins after which time the A_{405} of the mix was read against a blank of the reaction mix that had been incubated with 50µl distilled water.

2.21d. Dipeptidyl aminopeptidase assay

Total DPAP activity in a sample was assayed by adding the sample, in a 200μl volume, to 250μl 400mM Tris.Hepes (pH7.0) and 50μl of a 3mM solution in 25% methanol of the substrate X-pro-pNA and incubated at 37°C for 30 mins. The reaction was stopped by the addition of 500μl $ZnSO_4$ and 100μl 7.5% $Ba(OH)_2$, this led to the formation of a precipitate which was removed by centrifugation for 10 mins in a bench top microfuge. The A_{405} of the supernatant was read against a blank to which 200μl distilled water had been added in place of the sample. The activity of DPAP A (which is heat stable) was assayed by heating the sample to 65°C for 15 mins prior to its addition to the assay (being allowed to equilibrate to 37°C following this heat treatment) and the activity of DPAP B was estimated by subtracting the amount of heat stable DPAP activity in a sample from the total amount of DPAP activity in that same sample.

2.21e. NADPH:Cytochrome *c* oxidoreductase assay

Two identical aliquots of the sample to be assayed were each added, in 20μl volumes to 970μl of reaction mix containing 1.5mg/ml cytochrome c in 300mM potassium phosphate buffer (pH7.4) in matched quartz cuvettes. 10μl NADPH (16mg/ml in the phosphate buffer) was added to one of these samples and the change in A_{550} was followed.

2.21f. Assays for α-factor

Two assays were used for the detection of the mating pheromone; both are based on the inhibition of growth of a yeast strain that is sensitive to α-factor (RC631).

2.21fi. Halo assay for secreted α-factor

A growing culture of RC631 was diluted to a density of 10^6 cells/ml in YPD containing 0.8% (w/v) Bacto agar (no warmer than 50°C) and was poured immediately onto a YPD plate to create a lawn of sensitive cells. After the plate had been dried the strains to be tested for α-factor secretion were patched onto the seeded lawn (this was achieved by harvesting 10ml of a growing culture of the strain, resuspending it in as small a

volume as possible and dropping this onto the lawn, taking care that the plates had dried before continuing with the procedure). The plate was incubated at 25°C for approximately 36 hours, until the sensitive strain had grown and halos of growth inhibition could be seen around patches of yeast cells known to secrete α-factor (e.g. JRY188).

2.21fii. Bioassay for secreted α-factor

A growing culture of RC613 was diluted to a density of approximately 10^5 cells/ml using YPD. 100μl of this was added to 100μl of the supernatant to be assayed for the presence of the pheromone (or a suitable dilution of the same) contained within a well of a microtitre assay plate. The plate was incubated overnight (for approximately 18 hours) at 30°C after which the density of the sensitive cells was measured by removing the contents of the well from the plate and diluting it with 800μl distilled water in a 1ml cuvette and measuring the OD_{600} of this. A lower OD_{600} corresponds to a higher concentration of α-factor in the supernatant.

2.21g. Determination of protein concentrations

Protein concentrations were estimated using the method of Bradford (Bradford, 1976) using solutions containing known concentrations of BSA as standards.

2.22. Digestion of proteins using proteinase K

Proteinase K was added to samples to be treated (containing 2mM $CaCl_2$) to 50μg/ml. Digestion was allowed to proceed for 60 mins (on ice), after which the reaction was stopped by the addition of PMSF (to 3mM).

Chapter 3

The use of bacterially-synthesised hybrid proteins in the production of affinity-purified antibodies that specifically recognise Kex2p

3.1. Introduction

Central to the development of an immunoisolation procedure is the availability of an antibody that will bind to an antigen located in the structure of interest. For the purposes of this project antibodies that specifically recognise the cytoplasmically-disposed C-terminal domain of Kex2p were required.

In this project bacterially-produced fusion proteins were used to raise antibodies against various yeast proteins including Kex2p. This strategy of raising antibodies was chosen over the immunisation of rabbits with the proteins purified from yeast since it obviates the need to purify the various antigens and offers the advantage that antibodies against specific domains of the individual proteins may be obtained. In order to raise antibodies against various yeast proteins as part of this study, a gene fusion was constructed consisting of the part of the yeast gene encoding the region of the protein against which antibodies were desired, fused to a portion of DNA encoding IgG-binding domains of *S. aureus* protein A (Uhlen *et al.*, 1984). The gene fusions were then expressed in bacteria and the resulting protein products were purified and used as immunogens. Such a stategy was chosen for a number of reasons. Firstly, the IgG binding domains of protein A (included in the fusion protein) allow affinity purification of the protein (Nilsson *et al.*, 1985). This affinity of protein A for IgG (Langone, 1982) is also useful in screening for the synthesis of the fusion protein using immunoblot analysis. Also, it is thought likely that the repetitive structure of the protein A moeity enhances the immune response to the fusion protein (Lowenadler *et al.*, 1986).

This chapter describes, in detail, the production of affinity-purified antibodies that specifically recognise the cytoplasmically disposed C-terminal domain of Kex2p for use in immunoisolation of the yeast Golgi. Included here, as an appendix to this chapter, is a description of the production of antibodies against other yeast proteins, that are also used in this work.

3.2. The Spa-Kex2C fusion protein and its use as an immunogen

The use of gene fusions made it possible to ensure that only antibodies against the C-terminus of Kex2p were raised by ensuring that this was the only portion of the protein that the immune system of the rabbit was exposed to.

The plasmid pKpra (Figure 3-1) carries a segment of DNA (derived from the *spa* gene) encoding two of the IgG-binding domains of protein A and has a multiple cloning site to allow the insertion of DNA fragments adjacent to this. The *spa-KEX2C* gene fusion had been created by P. Whitley in the plasmid pKpraKex2C (Figure 3-1). This plasmid was constructed by cloning the 1.25kb fragment liberated upon digestion of pGA714 (Figure 3-1) with *Eco*RI and *Bam*HI into pKpra to create an in-frame gene fusion predicted to encode a protein A fusion protein (Spa-Kex2C) with a molecular weight of 29K.

The *spa* DNA contained within pKpra is under control of the *E.coli lac* promoter, expression from which can be induced by the presence of IPTG (Brown, 1991). The two plasmids pKpra and pKpraKex2C were transformed separately into the *E.coli* strain NM522 to create NM522(pKpra) and NM522(pKpraKex2C) respectively. The effect of the addition of IPTG to growing cultures of these strains upon their pattern of protein synthesis was investigated. Figure 3-2 shows that, as expected, pKpra encodes a protein with an apparent molecular weight of 23K (220 residues) which reacts with rabbit IgG, and that pKpraKex2C encodes a protein A fusion protein with an apparent molecular weight of about 40K. The Spa-Kex2C fusion protein is predicted to have a molecular weight of 29K (made up of 165 residues encoded by *spa* DNA and 100 residues encoded by the portion of *KEX2* used to create the gene fusion), but the protein detected in Figure 3-2 has an apparent molecular weight more than 10K higher than this. This discrepancy can be explained by the high net negative charge carried by the amino acid sequence of the C-terminal tail of Kex2p. When this portion of the protein is deleted the resulting shift in mobility of Kex2p in SDS polyacrylamide gels is greater than expected (Fuller *et al.*, 1989). From this, it is reasonable to accept that the

use of this portion of Kex2p in the construction of Spa-Kex2C has caused the fusion protein to run aberrantly in SDS polyacrylamide gels.

When large amounts of recombinant proteins are expressed in *E. coli*, as from the *lac* promoter, large insoluble aggregates of protein, known as inclusion bodies, often form (Maniatis *et al.*, 1982). In order to take advantage of protein A's affinity for IgG in the purification of Spa-Kex2C it was important to determine whether any of the fusion protein was present in a soluble form available for binding to IgG. This was investigated using differential centrifugation to remove insoluble material from lysed cells taken from a bacterial culture that had been induced to synthesise Spa-Kex2C and screening the various fractions obtained by this procedure for the presence of the fusion protein. As can be seen from Figure 3-3, a substantial amount of the fusion protein encoded by pKpraKex2C is found in the soluble fraction of a cell lysate. On the basis of this observation it was decided that IgG-Sepharose should be used in the purification of Spa-Kex2C. Comparison of Figure 3-3a with Figure 3-4a (i.e. comparison of a Coomassie-stained polyacrylamide gel of the soluble fraction prepared from a culture of NM522(pKpraKex2C) producing the fusion protein with that of the material recovered from this using IgG-Sepharose) shows that although the use of IgG-Sepharose has been successful in recovering Spa-Kex2C the sample obtained also contains other proteins.

It has been ascertained that, as indicated on Figure 3-4a, two of the contaminating high molecular weight proteins are DnaK and GroEL (Ellis and van der Vies, 1991 - this was shown to be the case by using affinity-purified antibodies, supplied by J. Zueco, against both proteins in immunoblot analysis, data not shown) and that another contaminating protein is the heavy chain of IgG (confirmed using the observation that this protein reacts with anti-rabbit IgG antibody in immunoblot analysis, data not shown) which has become detached from the Sepharose during elution of the bound material. The lower molecular weight contaminants are degradation products of the fusion protein, as suggested by the fact that they react with rabbit IgG in immunoblot

analysis (data not shown). Due to the presence of these contaminating proteins it was decided to include preparative SDS-PAGE as a final purification step in order to obtain a homogeneous preparation of the fusion protein (Figure 3-4b) for the immunization of rabbits.

The purified fusion protein was injected into rabbits from which immune sera putatively containing antibodies directed against the C-terminal domain of Kex2p were collected. Starting with a 500ml culture of NM522(pKpraKex2C) a typical yield of Spa-Kex2C fusion protein obtained by following the procedure laid out here would be 800-1000μg (as determined by the method of Bradford).

Figure 3-1
Creation of the spa-KEX2C gene fusion

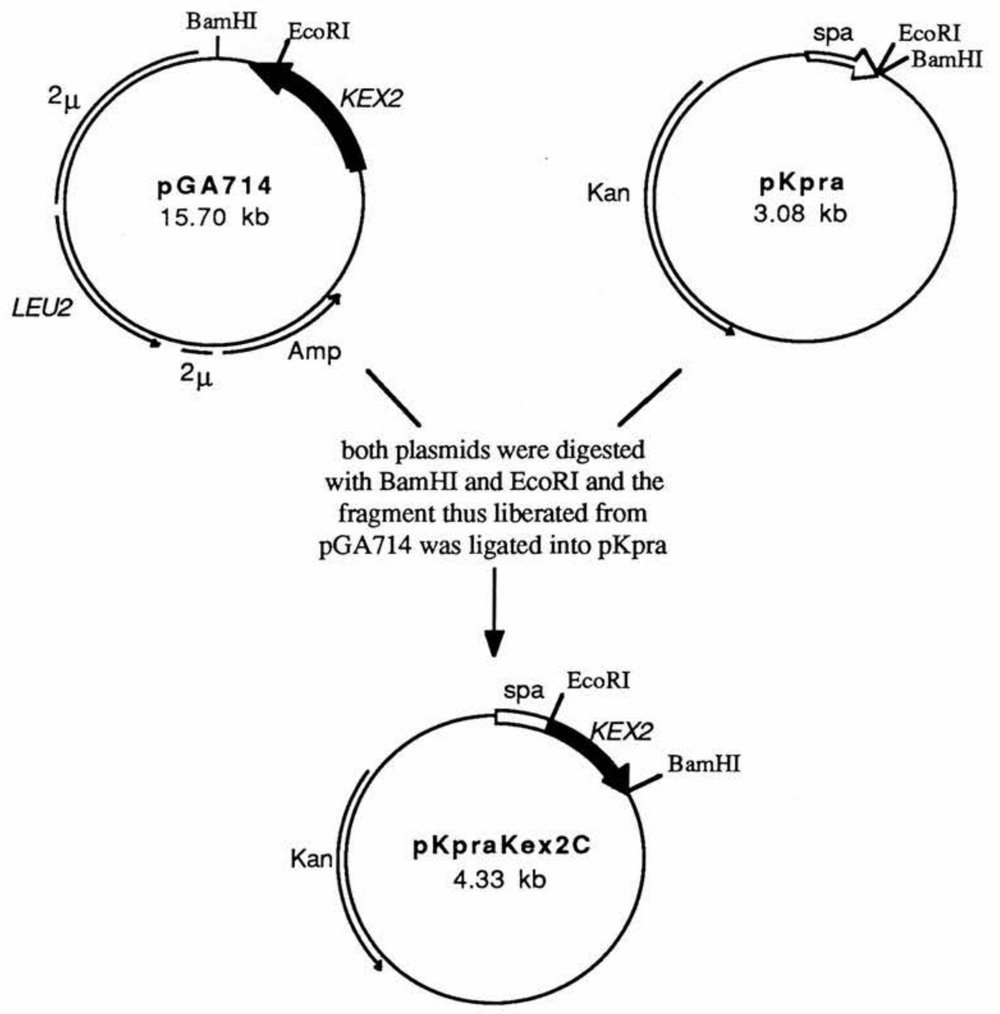


Figure 3-2

A protein A fusion protein produced by NM522(pKpra-KEX2C)

100ml cultures of both NM533(pKpra) and NM522(pKpraKEX2C) were grown in selective medium (L-broth containing 50μg/ml kanamycin) at 37°C to an OD_{600} of 0.8. Both cultures were then split in half. IPTG was added to one half of each culture to a final concentration of 0.25mM and the cultures were all left growing at 37°C for a further four hours. The half of each culture to which IPTG had been added was labelled as 'the induced culture'. 1.0ml samples were taken from each of the now four cultures. Cells were harvested from these and boiled in 100μl SDS sample buffer for 5 mins. The cell extracts thus prepared were analysed by 12% SDS-PAGE and immunoblot analysis using a rabbit IgG-HRP conjugate.

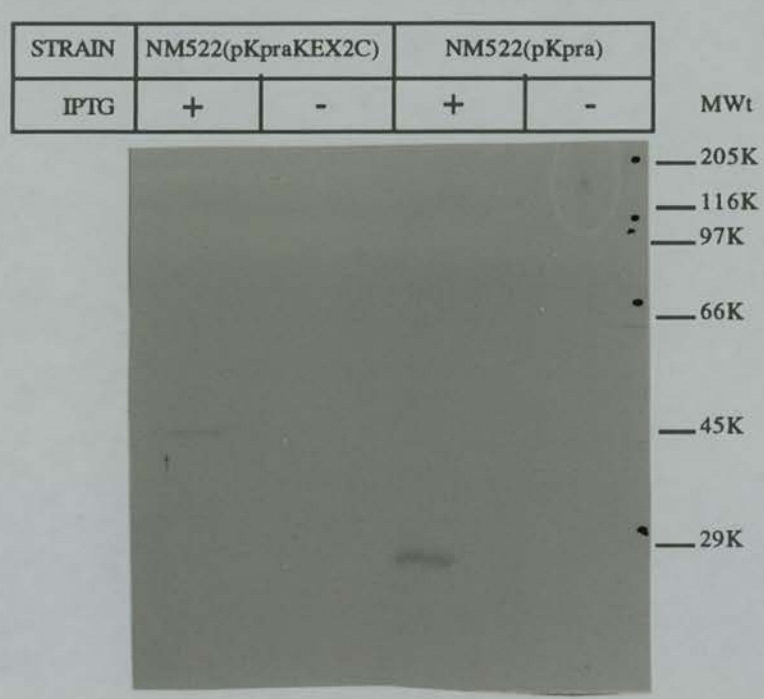

STRAIN
NM522(pKpraKEX2C)
NM522(pKpra)
IPTG
+
-
+
-
MWt
205K
116K
97K
66K
45K
29K

Figure 3-3

Analysis of an induced culture of NM522(pKpraKEX2C)

Cells from a 50ml 'induced culture' - (see Figure 3-2 legend) - were harvested, washed with 10ml Tris.HCl (pH7.4) and resuspended in 10ml of the same. Lysozyme was added to a final concentration of 1mg/ml and the cells were left on ice for twenty minutes. Cell lysis was achieved using sonication (4 x 30 seconds on ice with two minute intervals on ice to avoid excess heating). A 100μl sample was taken and labelled as 'whole cell extract' (WCE) before unlysed cells and cell debris were removed by centrifugation at 300g for 5 mins. The pellet from this was labelled as 'P3' and the supernatant was centrifuged for 20 mins at 25 000g to clear the cell lysate of any insoluble material yielding a soluble fraction, the 'S25', and 'the inclusion body preparation' or 'P25'. Both the P3 and the P25 were resuspended in 10ml Tris.HCl (pH7.4). All of the fractions obtained using the above procedure were run on two identical 12% polyacrylamide gels. One of these was stained with Coomassie blue (Figure 3-3a) and the other was used to transfer the samples to nitrocellulose to allow immunoblot analysis using a rabbit IgG-HRP conjugate to be performed (Figure 3-3b).

Figure 3-3a

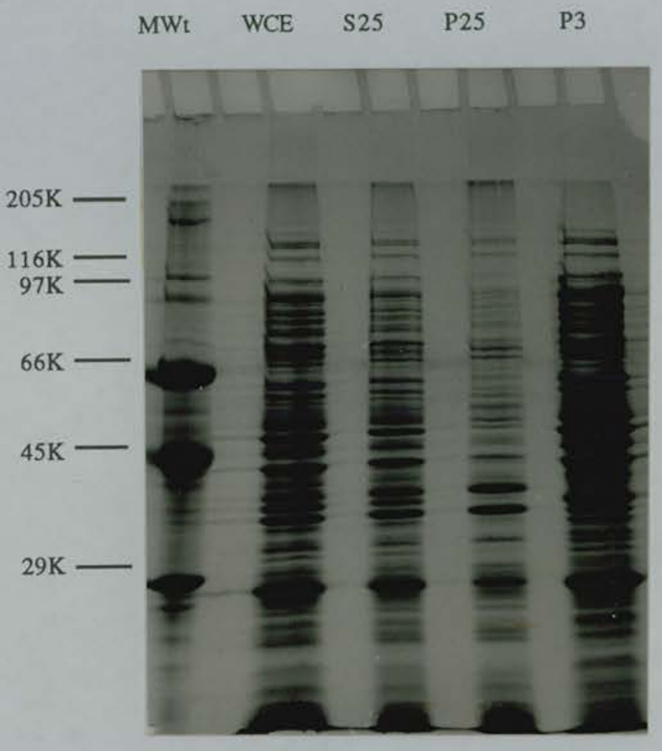


Figure 3-3b

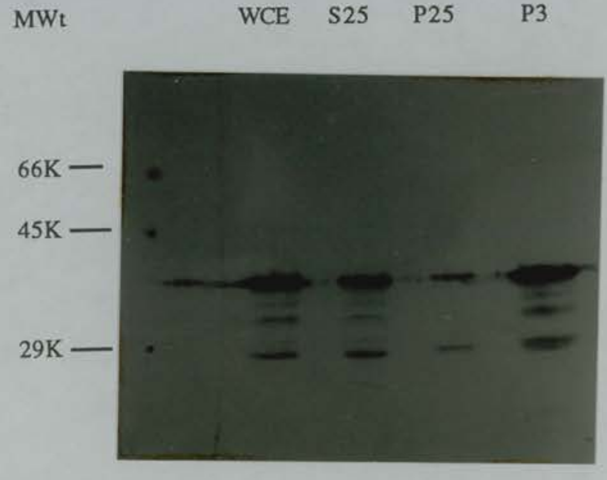


Figure 3-4

Purification of the Spa-Kex2C fusion protein

A 500ml culture of NM522(pKpraKex2C) was induced to express the Spa-Kex2C fusion protein as described in the legend for Figure 3-2. A soluble fraction was prepared from this culture (see the legend for Figure 3-3 for details). 1ml (slurry volume) IgG-Sepharose was added to this and left on a rotating wheel at 4^oC overnight. The IgG-Sepharose was recovered and washed six times in TST to remove non-specifically bound material. Elution of the bound material was achieved by washing the IgG-Sepharose (once for 20 mins and once for 5 mins) with 0.5M HAc(CH_3COOH) adjusted to pH3.4 with NH_4Ac. These washes were pooled and dialysed against distilled water overnight at 4^oC, before being freeze dried and resuspended in 0.5ml distilled water. An aliquot (10µl) of this was subjected to 12% SDS-PAGE and stained with Coomassie blue (Figure 3-4a). An identical gel was used to transfer the samples to nitrocellulose and the band indicated as Spa-Kex2C was shown to react with rabbit IgG by immunoblot analysis (data not shown).

The fraction eluted from the IgG-Sepharose was run on a 12% preparative SDS-poly acrylamide gel. The gel was lightly stained using Coomassie blue and a strip of the gel containing the band corresponding to the Spa-Kex2C fusion protein was cut out and placed in sealed dialysis tubing along with 5ml electrophoresis buffer. The protein was electroeluted (100mV for 5 hours) into the buffer contained within the dialysis tubing. This buffer was dialysed against distilled water before being freeze-dried. The lyophilised material was resuspended in 0.5ml distilled water. An aliquot of this (10µl) was subjected to 12% SDS-PAGE and stained with Coomassie blue (Figure 3-4b). The band indicated as the Spa-Kex2C fusion protein on Figure 3-4b was shown to react with rabbit IgG by immunoblot analysis (data not shown).

Figure 3-4a

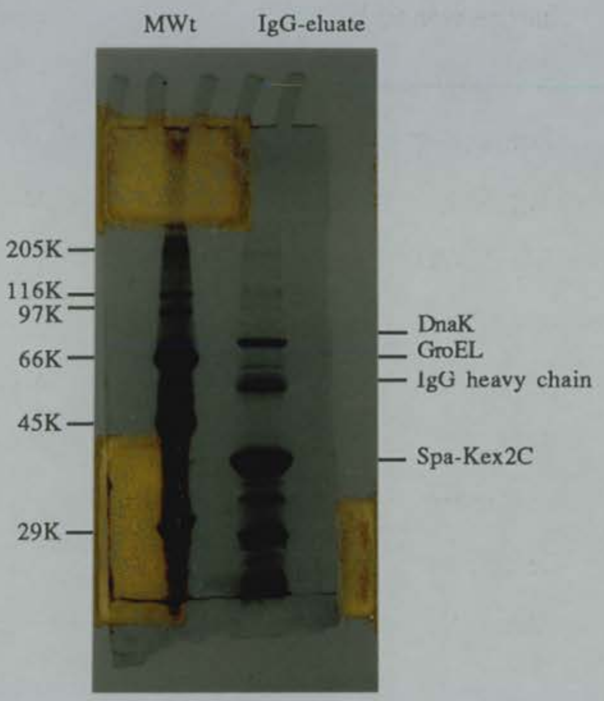
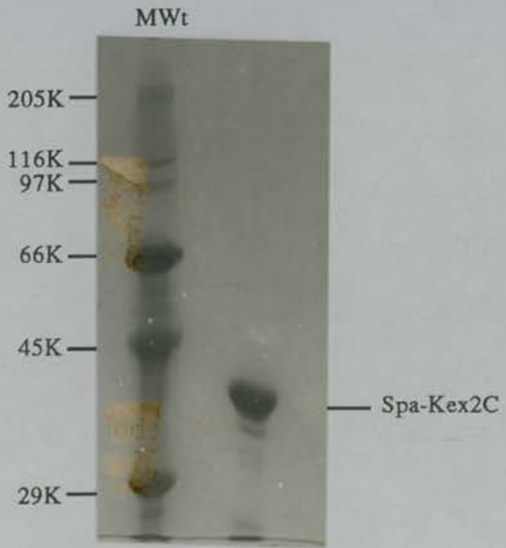


Figure 3-4b



3.3. Production of a βgal-Kex2C fusion protein

When working with polyclonal antiserum it is advantageous to be able to separate antibodies that specifically recognise the antigen of interest from other components of the serum. This is because the serum will contain antibodies that may recognise and bind to other proteins present in the preparation containing the antigen, and this would obviously be undesirable in an antibody preparation to be used for immunoisolation. Immunisation of a rabbit with Spa-Kex2C allowed immune serum containing antibodies that recognise the C-terminal 100 amino acids of Kex2p to be collected. In order to isolate these antibodies from the immune serum, a second Kex2p fusion protein was prepared. This time, the same portion of Kex2p that had been used in the construction of Spa-Kex2C was fused to βgal creating a βgal-Kex2C fusion protein. This was achieved by using the fragment of DNA from pGA714 (Figure 3-1) that had been used in the construction pKpraKex2C to create an in-frame gene fusion with the *lacZ* gene in the plasmid pEX11 (Figure 3-5). This gene fusion resulted in the construction of the plasmid pNB21 and was predicted to encode a βgal-Kex2C fusion protein (βgal-Kex2C) with a molecular weight of 127K.

Transcription of the *lacZ* gene in the pEX vector family, of which pEX11 is a member, is under control of the P_R promoter of bacteriophage lambda (Kusters *et al.*, 1989). Regulated expression can be achieved in a host strain of *E.coli* (such as pop2136) which harbours lysogenised lambda carrying the thermosensitive *c*I857 allele. At 30°C the repressor is functional whereas at 42°C it is not; this means that a shift to 42°C of a growing culture of pop2136 cells containing a pEX vector allows expression of the gene under control of the P_R promoter.

pop2136 cells were transformed separately with both pEX11 and pNB21 creating pop2136(pEX11) and pop2136(pNB21) respectively. Growing cultures of both these strains were shifted from 30°C to 42°C for four hours. Figure 3-6 examines the effect of such a temperature change upon the pattern of proteins produced by these strains.

There is a protein present in the extract prepared from pop2136(pEX11) cells that had been shifted to 42°C, which is not present in that prepared from cells that had been left at 30°C, corresponding to the pEX11 βgal product (117K). Similarly, there is a protein present in the lane containing pop2136(pNB21) extract prepared from cells that had been shifted to 42°C which is not present when the same cells were left at 30°C and this band is taken to be the βgal-Kex2C fusion protein (127K).

Examination of cells producing the βgal-Kex2C fusion protein by light microscopy suggests that they contain inclusion bodies (they adopt a filamentous morphology with a refractile body at one end: both of these characteristics are typical of cells containing inclusion bodies). These inclusion bodies were partially purified using differential centrifugation to separate them from soluble material present in the cells, and immunoblot anlysis using affinity-purified anti-βgal antibody (supplied by J. Zueco) as primary antibody was carried out on the various fractions yielded by this procedure. Figure 3-7 demonstrates that essentially none of the βgal-Kex2C fusion protein is present in a soluble form in the cell extract.

βgal-Kex2C is easily identifiable on a Coomassie-stained gel in the lane containing whole cell extract prepared from an induced culture of pop2136(pNB21)-(Figure 3-7a). It was decided that preparative SDS-PAGE should be used to obtain a sample of the fusion protein from such cell extracts since attempts at solubilizing the inclusion bodies (Maniatis *et al.*, 1982) with the eventual aim of purifying the fusion protein by affinity chromatography proved unsuccessful.

Whole cell extract prepared from an induced culture of pop2136(pNB21) was subjected to electrophoretic separation on a 10% polyacrylamide gel. After Coomassie-staining, a gel slice containing the fusion protein was excised and the protein was recovered from this by electroelution.

3.4. Affinity purification of anti-Kex2C specific antibodies

βgal-Kex2C purified as described was bound to Affi-gel 10 (Frost *et al.*, 1981) and used to purify antibodies that specifically recognise the C-terminal domain of Kex2p from immune sera raised against the Spa-Kex2C fusion protein. Routinely, from 5ml immune sera obtained from a rabbit that had been immunised using Spa-Kex2C approximately 25mg (as estimated by the method of Bradford) of affinity purified antibody (anti-Kex2C) was obtained. The specificity of anti-Kex2C is demonstrated in Chapter 4.

Figure 3-5
Construction of the β gal-KEX2C gene fusion

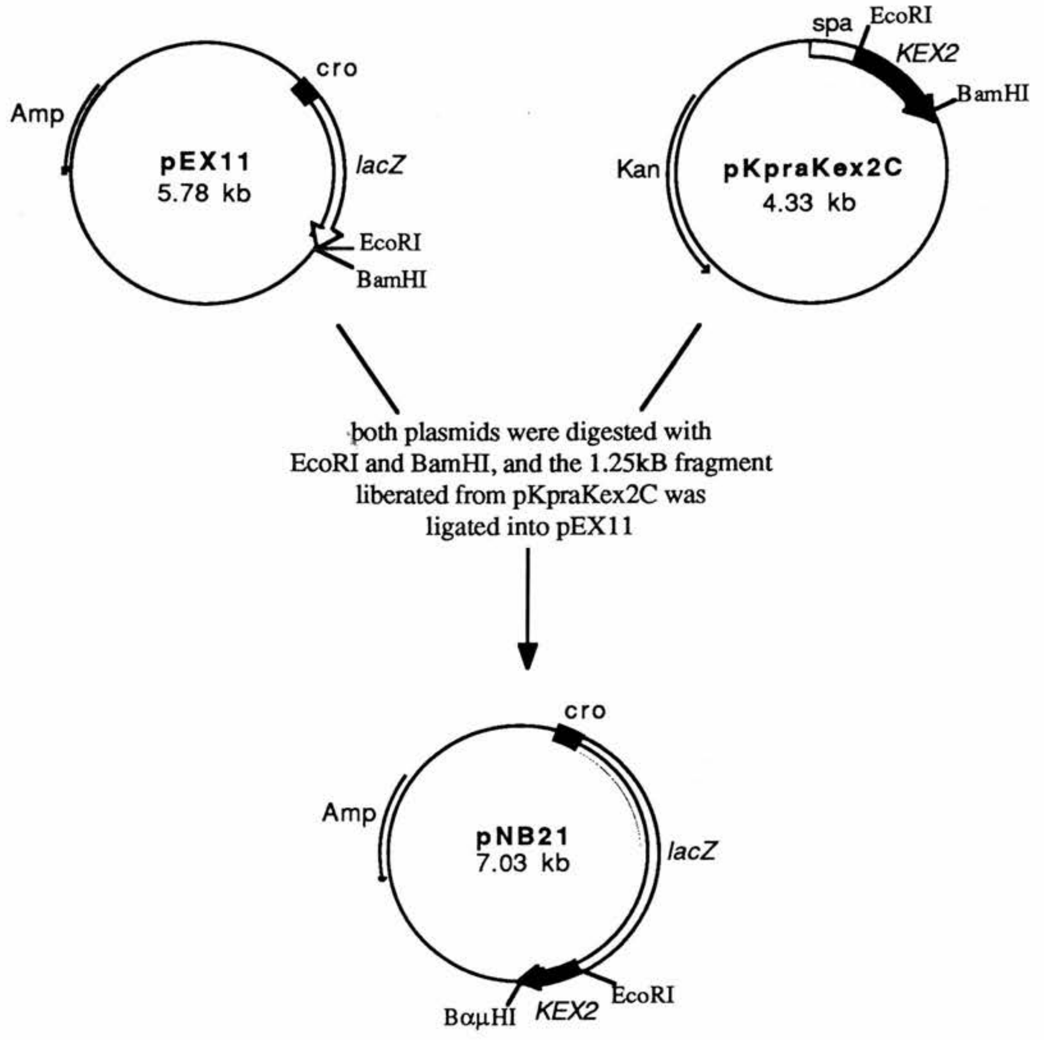


Figure 3-6

Proteins produced by pop2136(pNB21)

100ml cultures of pop2136(pEX11) and pop2136(pNB21) were grown at 30°C in selective medium (L-broth containing 100µg/ml ampicillin) to an OD_{600} of 0.5. Both cultures were then split in half with 50ml of each being left at 30°C and the other 50ml of each being moved to 42°C for four hours. The half of each culture that was moved to 42°C was labelled at the 'induced culture'. 1.0ml aliquouts were taken from the now four cultures and the protein composition of cells from these were analysed using 10% SDS-PAGE with the resultant gel being stained with Coomassie blue.

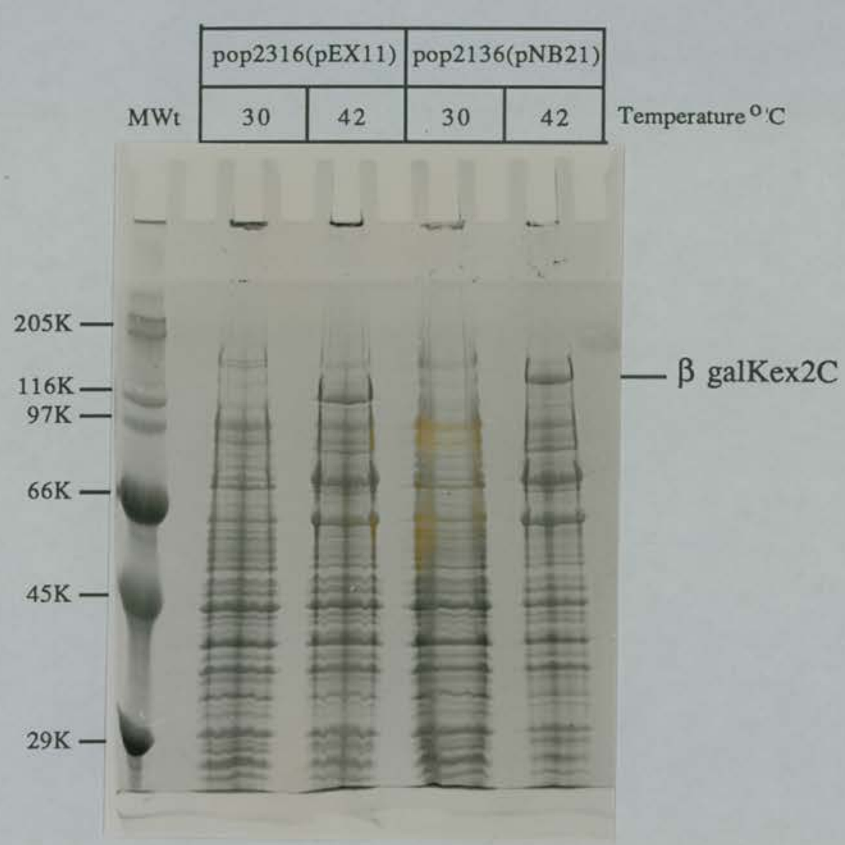

pop2316(pEX11)
pop2136(pNB21)
MWt
30
42
30
42
Temperature °C
205K
116K
97K
66K
45K
29K
β galKex2C

Figure 3-7

A βgal-Kex2C fusion protein produced by pop2136(pNB21)

Cells from a 50ml culture of pop2136(pNB21) which had been induced to produce the βgal-Kex2C fusion protein as in the legend for Figure 3-6 were treated in the same way as those from an induced culture of NM522(pKpraKex2C) were as described in the legend for Figure 6-3 yielding whole cell extract (WCE), P3 (unlysed cells and cell debris), P25 (inclusion bodies), and S25 (soluble material). These fractions were run on two 10% polyacrylamide gels one of which was stained with Coomassie blue (Figure 3-7a) with the other being used to transfer the samples to nitrocellulose for immunoblot analysis using affinity-purified anti-βgal antibody as primary antibody (Figure 3-7b).

Figure 3-7a

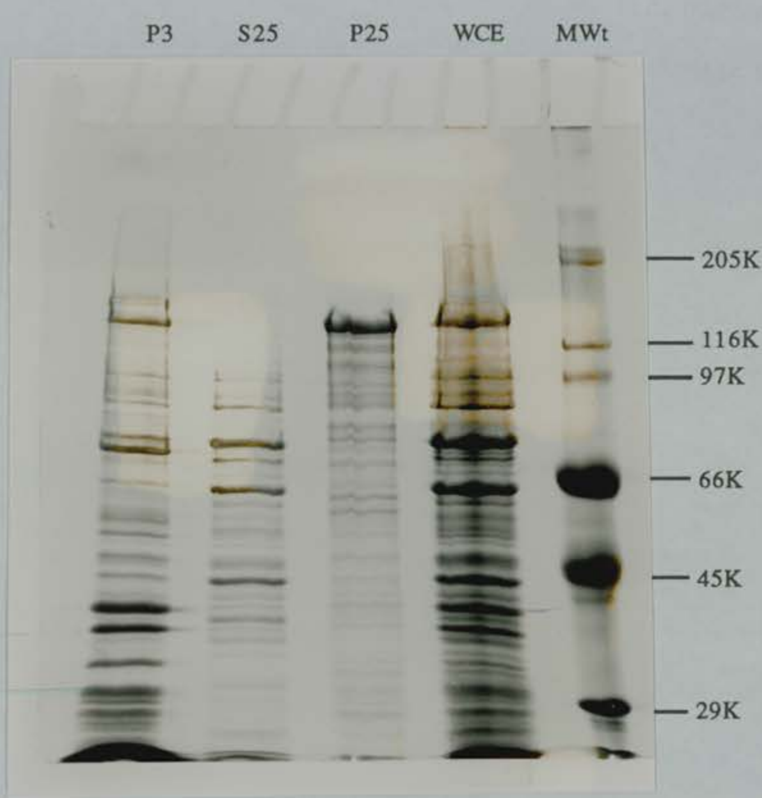
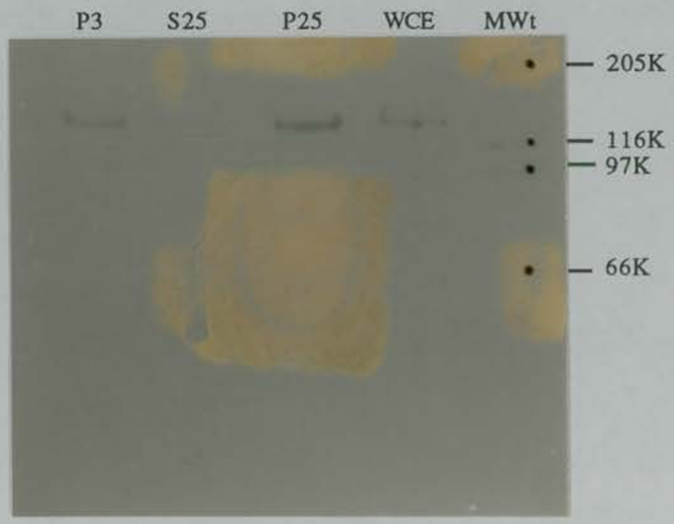


Figure 3-7b



Appendix to Chapter 3

Other antibodies produced during the course of this work

3A.1. Introduction

In addition to the anti-Kex2 antibody, it was desirable to obtain antibodies against other yeast membrane proteins in order to evaluate the purity of material immunoisolated using the anti-Kex2p antibody. To demonstrate the purity of a membrane fraction it is necessary to show that it is free from contamination by membranes derived from organelles other than the one of interest. This can be achieved by showing that the fraction does not contain activities of enzymes associated with potential contaminating organelles. However, such information can be misleading since few enzyme assays are absolutely specific for the particular marker enzyme that is being monitored. For example, the assay used throughout this study to follow Kex2 protease activity does not detect the activity of just the Kex2 protease. Cell extracts prepared from yeast cells in which the *KEX2* gene has been disrupted (PWYS3) cleave the fluorogenic peptide used in the assay albeit at a rate twenty times slower that an extract prepared from cells in which the *KEX2* gene is intact (JRY188). The *KEX2* gene of PWYS3 has been disrupted in such a way that the portion of DNA known to encode the catalytic domain of Kex2p has been removed. Immunoblot analysis performed on the same extracts using a polyclonal antibody raised against Kex2p detects a protein with an apparent molecular weight of 135K present in the extract prepared from JRY188 but not in that prepared from PWYS3. From these results (Whitley, 1990), it can be concluded that the cleavage of the peptide by the PWYS3 extract is due to an activity distinct from that of Kex2p.

Antibodies that recognise a number of yeast proteins were raised as part of this work, and their production is described in this appendix. In each case, methods similar to those used for the production of the anti-Kex2C antibodies were used.

3A.2. Antibodies that recognise Ste2p

3A.2a. Preparation of anti-Ste2p antibodies

Antibodies were prepared against the product of *STE2* (the receptor for the mating pheromone α-factor - Burkholder and Hartwell, 1985), for two reasons. Firstly, these antibodies were to be used to demonstrate whether material immunoisolated using the anti-Kex2C antibody contained any plasma membrane derived material (the receptor is an integral membrane protein located in the plasma membrane). Secondly, the antibodies were raised specifically against the cytoplasmically-disposed C-terminal domain of the protein so that they could be of use in the future to immunoisolate inside-out plasma membrane vesicles (such vesicles could be used to develop a protein transport assay involving the fusion of post-Golgi secretory vesicles with the plasma membrane). A *spa-STE2* gene fusion was created by cloning a portion of DNA (about 400 base pairs) from the coding region of the *STE2* gene (Nakayama *et al.*, 1985) contained within the plasmid pAB510 into pKpra (Figure 3-1). In order to create an in-frame gene fusion as required the cloning strategy outlined in Figure 3A-1 was undertaken. It was predicted that the *spa-STE2* gene fusion contained within pNB4 should encode a protein A fusion protein with a molecular weight of 40K (comprised of 165 residues encoded by pKpra DNA and 135 residues encoded by *STE2* DNA) and this was shown to be the case (data not shown). It was ascertained that the Spa-Ste2p fusion protein produced by NM522(pNB4) did not form inclusion bodies (data not shown) and therefore it was possible to purify the fusion protein using IgG-Sepharose. The purified fusion protein was injected into a rabbit from which immune serum was subsequently collected.

In order to separate antibodies specific to the C-terminal 135 residues of Ste2p from other antibodies present in immune serum raised against the Spa-Ste2p fusion protein, a βgal-Ste2p fusion protein was prepared whereby the same 135 residues of Ste2p that were used in the construction of Spa-Ste2p were fused to βgal. This was achieved by cloning the same portion of DNA from pAB510 that was used in the construction of

pNB4 into pEX11 to create the plasmid pNB13 containing an in-frame gene fusion. pNB13 is predicted to, and was shown to, encode a βgal fusion protein of molecular weight 131K (data not shown). This fusion protein was purified from a culture of pop2136(pNB13) and was used to affinity purify antibodies specific to the C-terminal 135 amino acids of Ste2p (anti-Ste2p).

3A.2b. Demonstration of the specificity of anti-Ste2p

In order to demonstrate the specificity of anti-Ste2p for the pheromone receptor, a yeast strain containing a unique, inducible copy of the *STE2* gene was created. In order to create such a strain the plasmid pIH2-4, which harbours a copy of *STE2* under the control of the *GAL1* promoter, was transformed into the diploid yeast strain, NBY10 (in which haploid specific genes under mating type control are not expressed; i.e. it does not produce Ste2p, or α-factor) to create NBY10(pIH2-4).

Two cultures of NBY10(pIH2-4) were grown: one using galactose as a carbon source, (the cells in this culture will produce Ste2p); and the other using glucose as a carbon source. The cells in the culture which had been grown on glucose will not express *STE2* because such growth conditions repress the *GAL1* promoter.

Due to the hydrophobicity of Ste2p (it contains seven transmembrane spans) it was found that the protein could not be detected using anti-Ste2p against yeast cell lysates that had been boiled in SDS sample buffer prior to separation by SDS-PAGE and transfer to nitrocellulose (a procedure often used for the preparation of samples to detect proteins in this way). Instead, a crude preparation of membranes was obtained from each culture and solubilised with detergent before being subjected to SDS-PAGE and immunoblot analysis (Konopka *et al.*, 1988) (N.B. it was found that the protein could only be detected by immunoblot analysis if buffers not containing methanol were used throughout the transfer of proteins to the nitrocellulose filter). As can be seen from Figure 3A-2 the affinity-purified antibodies react with the sample prepared from the

cells supplied with galactose as their sole carbon source but not with that prepared from cells that had been exposed to repressing concentraions of glucose.

From the published sequence of the *STE2* gene the predicted molecular weight of the protein is 47 794. Figure 3A-2 shows that the antibody recognises two bands in the sample prepared from the culture that had been induced to produce Ste2p which are not present in the sample prepared from cells which had not been induced to produce the protein, this pattern is similar to that found in another study involving the detection of Ste2p by immunoblot analysis (Konopka *et al.*, 1988). The lower of the two bands has an apparent molecular weight of between 50 and 55kD and is taken to be the monomeric form of STE2p. One reason for the fact that this is of a higher apparent molecular weight than might have been expected from the gene sequence is that the protein is glycosylated. The higher molecular weight material migrates to an area of the gel corresponding to about 100kD and may be accounted for by the likelihood that aggregates of the protein may not be completely broken down since the samples are not boiled.

Figure 3A-1

Construction of the *spa-STE2* gene fusion

(1) The 1.6kb fragment, containing the *STE2* gene, yielded upon the digestion of pAB510 with *Hin*dIII was cloned into the *Hin*dIII site of pK19 to create pNB1. Clones containing the *Hin*dIII fragment in the desired orientation were identified as those which a) produced a 1.6kb fragment upon digestion with *Hin*dIII and b) produce a 1.6kb fragment upon digestion with *Sal*I.

(2) The largest *Hae* III fragment (0.8kb) from pNB1, which is also the only *Hae*III fragment from pNB1 that contains a *Sal*I site, was cloned into the *Sma*I site of pUC18 to create pNB2. Clones containing the correct fragment in the desired orientation were taken as those which could be digested with *Hin*dIII to give a 0.6kb fragment and with *Sal*I to give a 0.3kb fragment.

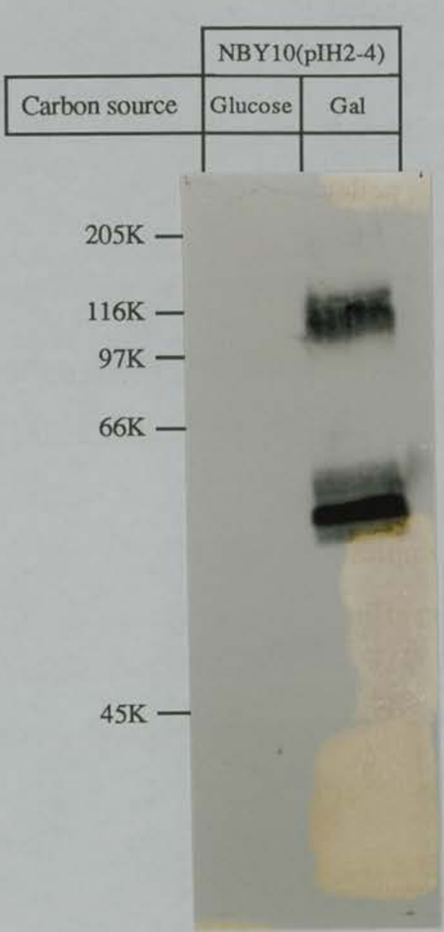
(3) pNB2 was digested with *Kpn*I and *Bam*HI and the 0.8kb fragment thus liberated was directionally cloned into pKpra to create pNB3 with positive clones being identified as those which a) released the cloned *Bam*HI, *Kpn*I fragment upon digestion with the two enzymes, b) are linearised upon digestion with each of *Sal*I, *Pst*I and *Hinc*II, and c) yield two fragments, of 2.8kb and 1.0kb, upon digestion with *Hin*dIII, the smaller of which contains both a *Pst*I and a *Hinc*II site.

(4) pNB3 was linearised by digestion with *Sal*I and the cohesive ends thus created were filled in using Klenow fragment. A blunt end ligation created pNB4 containing a *spa-STE2* gene fusion. Positive clones were screened for by checking for the disappearance of the *Sal*I site.

Figure 3A-2

Recognition of Ste2p by affinity purified anti-Ste2p antibodies

Two 50ml cultures of NBY10(pIH2-4) were grown in selective medium, one was supplied with galactose as its sole carbon source (Gal), and the other was supplied with glucose (Glucose). The cultures were grown to early log phase after which the cells from both were harvested, washed once with water, and resuspended in 0.5ml 10mM Tris.HCl (pH7.5), 1mM EDTA. The cells were disrupted by vortexing in the presence of glass beads and the resultant extracts were centrifuged (10 0000g) for 30 mins. The pellet thus obtained was extracted with 1% deoxycholate, 10mM Tris.HCl (pH7.5) on ice for 30 mins. Solubilised material was mixed with an equal volume of SDS sample buffer containing 8M urea (prewarmed to 65°C). The samples were incubated at 65°C for 10 mins before being resolved by electrophoresis on a 12% polyacrylamide gel and then transferred to nitrocellulose (it was important that no methanol was present in the buffers used during the transfer of the samples to nitrocellulose). Immunoblot analysis was performed on the filter using affinity-purified anti-Ste2p as primary antibody at a dilution of 1 in 1000.


NBY10(pIH2-4)
Carbon source
Glucose
Gal
205K
116K
97K
66K
45K

3A.3. Antibodies that recognise the yeast plasma membrane H^+-ATPase

Antibodies that specifically recognise a second yeast plasma membrane protein , Pma1p were also raised during the course of this project. Pma1p, the product of the yeast *PMA1* gene, is a H^+ -translocating ATPase (Serrano*et al.*, 1986).

The oligonucleotides 5'-CTCTTGGTGGATCCATACATGG-3' and 5'-TGGCCCGGGCTAAGAAACAAGCCATTGTTC-3' were used as primers for PCR to generate a fragment of DNA that encodes from Ala_{354} to Asp_{534} of Pma1p using genomic DNA prepared from NBY10 as template DNA. The product of this reaction (which had been shown to be the desired product, since it could be digested by *Eco*RI to give two fragments, 288bp and 273bp) was digested with *Sma*I and *Bam*HI and cloned into similarly digested pAX11 to form a *spa-PMA1* gene fusion contained within the plasmid pNB75, and also into pEX11that had also been digested with *Sma*I and *Bam*HI to form pNB76. The fusion protein encoded by the *spa-PMA1* gene fusion was purified from bacteria producing it (using IgG-Sepharose) and was used to immunise mice. It was decided to raise antibodies in mice in this case for two main reasons: Firstly, it has been reported that mouse antiserum tends to recognise fewer proteins in yeast non-specifically (V. Bankaitis - personal communication); and secondly mice are cheaper and easier to keep than rabbits.

The immune serum obtained from one of the mice was found to recognise the βgal-Pma1p fusion protein encoded by pNB76, but not the βgal-Kex2C fusion protein encoded by pNB11 (this was ascertained by immunoblot analysis using the serum as primary antibody against whole cell extracts prepared from induced cultures of pop2136(pNB76) and pop2136(pNB11) that had been electrophoretically separated on a 10% polyacrylamide gel - data not shown). This serum was then tested for reaction with a yeast cell lysate and with membrane and cytosol fractions prepared from this by differential centrifugation. Figure 3A-3 shows that the serum reacts with the crude lysate prepared from the yeast strain NBY10 and also with the membrane (P100) fraction, but not with the cytosolic (S100) fraction. Pma1p is predicted to have a

molecular weight of approximately 100K. Figure 3A-3a demonstrates that the antiserum reacts with an area of the immunoblot corresponding to proteins with apparent molecular weights ranging from approximately 90K to approximately 200K that are present in a cell lysate and enriched in the P100.

At the same time as blood from the immunised mice was obtained, the spleen from one was removed and used to produced hybridoma cell lines by J. Haywood. Culture supernatants from these cell lines were tested for reaction with yeast cell lysates. Primary screening was carried out by dot-blot analysis. Supernatants that reacted with yeast extract were then tested for reaction with the βgal-Pma1p fusion protein (using immunoblot analysis against bacterial whole cell extract prepared from an induced culture of pop2136(pNB76) that had been electrophoretically separated on a 10% polyacrylamide gel - data not shown). Those that recognised the βgal-Pma1p fusion protein, but showed no reaction with the βgal-Kex2C fusion protein were finally tested for recognition of a protein corresponding to Pma1p in a membrane fraction prepared from yeast cells. Figure 3A-3b shows the reaction of supernatant from the cell line B/1A1 with a protein present in the P100 prepared from a yeast cell lysate, which has an apparent molecular weight of approximately 150K. Such an apparent molecular weight is consistent with the protein recognised here being Pma1p (by comparison of the immunoblot shown in figure 3A-3b with one presented in Serrano *et al.*, 1986)

3A-3.

Immunoblotting of the yeast plasma membrane H^+-ATPase (Pma1p)

A cell lysate prepared from the yeast strain NBY10 was fractionated by differential centrifugation to yield a P100 and a S100. These were electrophoretically separated on a 10% polyacrylamide gel and were subsequently transferred to nitrocellulose. Figure 3A-3a shows an immunoblot that was performed against these samples using antiserum from a mouse that had been immunised with the Spa-Pma1p fusion protein as primary antibody, and anti-mouse IgG-HRP and anti-mouse IgM-HRP (both at a dilution of 1 in 3000) as secondary antibody.

Figure 3A-3b shows an immunoblot performed against a P100 and an S100 (as above) using supernatant from the cell line B/1A1 at a dilution of 1 in 5 in blocking buffer as primary antibody, and anti-mouse IgG-HRP (at a dilution of 1 in 3000) as secondary antibody.

Figure 3A-3a

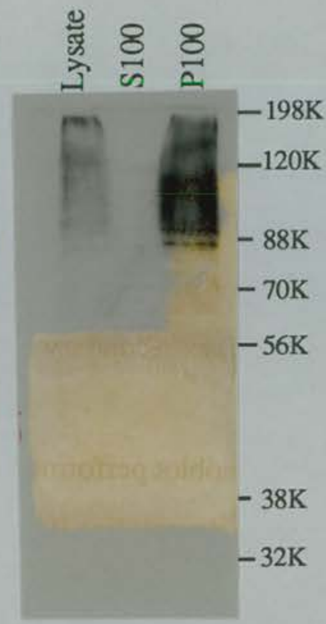
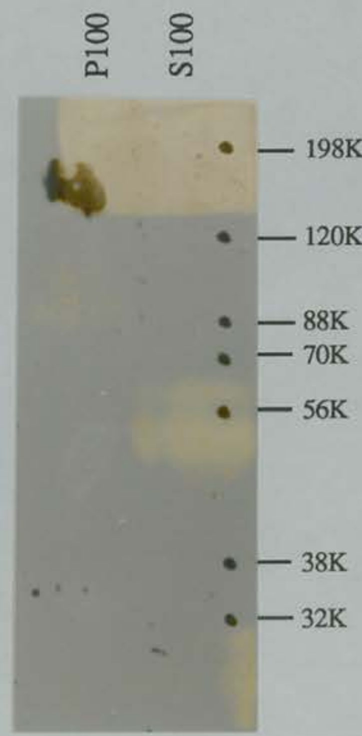


Figure 3A-3b



3A.4. Antibodies that recognise HMG-Coenzyme A reductase

PCR using the oligonucleotides 5'-GAATGCTGCTAGAATTCATACCAG-3' and 5'-CGGGATCCAATGACGTATGACTAAGTTTAGG-3' and genomic DNA prepared from NBY10 gave rise to a fragment of DNA that encodes part of the integral ER membrane protein HMG-CoA reductase (from Ile_{524} to Ser_{1054} - Basson *et al.*, 1988), encoded by *HMG1* (Hmg1p). The DNA obtained as a result of this reaction was shown to be the desired fragment by digesting it with *Hin*dIII, this yielded three fragments, 867bp, 548bp, and 22bp. The PCR product was cloned, as an *Eco*RI, *Bam*HI fragment, into the plasmid pAX12 to form pNB72, and also into pEX12 to create pNB73.

The Spa-Hmg1p fusion protein encoded by pNB72 was purified from an induced culture of NM522(pNB72) using IgG-Sepharose and was used to immunise a rabbit from which immune serum was subsequently collected. This serum was found to recognise the βgal-Hmg1p fusion protein encoded by pNB73, but not the βgal-Kex2C fusion protein (by using the serum as primary antibody in immunoblot analysis against induced cultures of pop2136(pNB73) and pop2136(pNB21) separated on a 10% polyacrylamide gel). *HMG1* encodes a protein with an apparent molecular weight of 115K (Wright *et al.*, 1988). It can be seen, from Figure 3A-4a that the immune serum raised against the Spa-Hmg1p fusion protein does not recognise a protein of this molecular weight in either a yeast cell lysate, or a P100 prepared from this when used in immunoblot analysis against these fractions.

Hmg1p is predicted to have seven transmembrane spans (Basson *et al.*, 1988), and therefore it was thought that the lack of detection of the protein with the antiserum may be due to problems similar to those encountered during initial attempts to detect Ste2p by immunblot analysis. A sample prepared using conditions that allow the detection of Ste2p was tested for reactivity with the antiserum. Figure 3A-4b shows that this has allowed the detection of some high molecular weight material that was not detected in a P100 that had been placed in a boiling water bath for five minutes prior to

electrophoretic separation. Attempts at affinity-puriying Hmg1p-specific antibodies using the βgal-Hmg1p fusion protein encoded by pNB73 proved unsuccessful, perhaps due to a low titre of such antibodies in the serum.

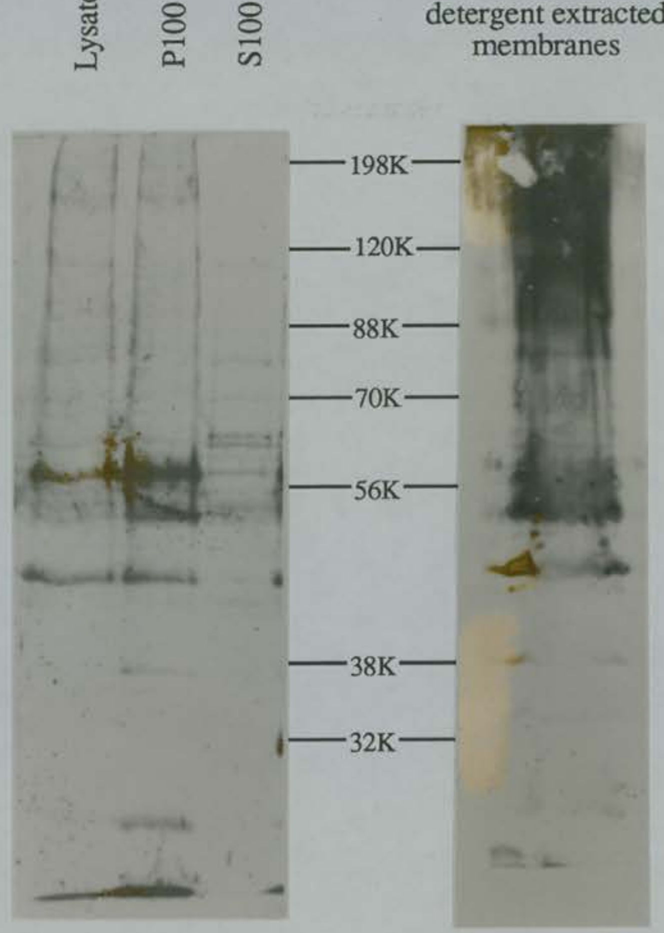
Figure 3A-4.

Detection of HMG-CoA reductase by immunoblot analysis

A cell lysate prepared from the yeast strain NBY10 was fractionated to yield a P100 and a S100. These were electrophoretically separated on a 10% polyacrylamide gel and transferred to nitrocellulose. Figure 3A-4a shows an immunoblot that was performed against these samples using antiserum from a rabbit that had been immunised with the Spa-Hmg1p fusion protein as primary antibody.

Figure 3A-4b shows an immunoblot that was performed using the same serum against a sample that had been prepared as described in the figure legend for Figure 3A-2 (i.e. a crude preparation of membranes extracted with detergent prior to electrophoretic separation).

Figure 3A-4a **Figure 3A-4b**



Chapter 4

The use of affinity-purified anti-Kex2p antibodies in the immunoisolation of Kex2p from yeast cells

4.1. Introduction

This chapter describes how antibodies directed against the cytoplasmically-disposed C-terminal domain of Kex2p (anti-Kex2C; preparation described in Chapter 3) were used to isolate membrane vesicles derived from the Kex2-containing compartment of the yeast Golgi. This was achieved using the technique of immunoisolation the theory of which is outlined briefly in Chapter 1.

The purity of material obtained through immunoisolation depends largely on the specificity of the antibody used in the formation of the immunoadsorbent (ImAd). It is therefore important to use an antibody which recognises only the antigen of interest in the preparation from which material is to be isolated. For this reason it is common for affinity purified antibodies to be used, since they tend to recognise less non-specific material than do crude sera. Figure 4-1 demonstrates that anti-Kex2C recognises only Kex2p in a yeast cell extract.

For an antibody to be of use in recovering material it is necessary for it to be attached to a solid support to allow recovery of any bound material from biological preparations. There are a number of matrix materials which may be used for this purpose. The matrix must be inert, showing no reaction with the system in which it is to be used, and obviously it must be capable of binding antibody molecules. For indirect immunoisolation (Chapter 1), agarose or magnetic beads carrying chemical groups (e.g. tosyl groups) that will covalently bind proteins under defined conditions can be used to preform the ImAd. Alternatively, a matrix carrying an antibody that will recognise and bind the antibody used for the immunoisolation may be used; if this method is used it is desirable that the second antibody recognises the Fc portion of the first, as this will ensure that the antigen binding sites of the first antibody are free to bind antigen. Such orientation of the antibody can also be ensured if binding to the matrix is achieved through the *S. aureus* coat protein, protein A, which has high affinity for the Fc portion of rabbit IgG molecules (Langone, 1982), but cannot be ensured if the antibody is attached to the

matrix through, for example, tosyl groups which will bind to any available amino group (Nilsson and Mosbach, 1984) in the antibody molecule and may lead to its being attached to the matrix through an antigen binding site, thus rendering it unable to bind antigen due to steric hinderance, and resulting in the formation of an inefficient ImAd. Matrices which bind antibody as a result of specific recognition, such as those carrying protein A, can be used in either direct or indirect immunoisolation (Chapter 1), whereas those which bind antibody molecules through amino groups are obviously restricted to use in direct immunoisolation. It was such considerations that led to the choice of matrix used here namely, fixed *S. aureus* cells (Pansorbin).

4.2. Preparation and use of a Kex2-Immunoadsorbent

Affinity purified anti-Kex2C was bound to Pansorbin to form a Kex2-immunoadsorbent (Kex2-ImAd). Rabbit IgG binds to protein A through the Fc portion of the immunoglobulin and therefore it is envisaged that the antigen binding sites of the antibody remain free to bind to any available antigen. Figure 4-2 demonstrates that Kex2-ImAd can be used to recover functional Kex2p from a cell lysate prepared from the yeast strain NBY10(pGA714), whereas none is recovered using Pansorbin to which no antibody has been attached. Kex2p recovered in this way is detectable by immunoblot analysis (Figure 4-2a), and the fact that the recovery can be blocked (Figure 4-3) by preincubation of the ImAd with βgal-Kex2C fusion protein (which will occupy antigen binding sites of antibodies that recognise the C-terminal domain of Kex2p) confirms that the observed recovery is mediated by specific antibody-antigen interaction. No such block of recovery is seen when the preincubation is carried out using an unrelated fusion protein, βgal-Ste2p. Since the two fusion proteins used carry the same βgal derived portion, it can be concluded that the inhibition of Kex2p recovery is due to the Kex2 derived portion of the βgal-Kex2C fusion protein occupying antigen binding sites present in the ImAd and thus destroying its ability to bind Kex2 protein from the cell lysate.

Figure 4-1.

Specificity of affinity-purified anti-Kex2C antibody

Glass bead extracts were prepared from growing cultures of NBY10, NBY10(pGA714) and PWYS3. These were subjected to electrophoretic separation on a 10% polyacrylamide gel and then transferred to nitrocellulose. Affinity-purified anti-Kex2C was used as primary antibody in immunoblot analysis of the lysates on this filter. This figure shows that anti-Kex2C recognises a single protein of apparent molecular weight 135K in extracts from NBY10 and NBY10(pGA714), but does not recognise any component of the PWYS3 extract (the *KEX2* gene of PWYS3 cells has been disrupted). The extract prepared from NBY10(pGA714) contains a higher amount of the 135K protein than that prepared from NBY10 (an equivalent amount of protein from the two extracts was loaded on the gel that was used for this immunoblot), this is due to the presence of pGA714 which causes the cells to overexpress Kex2p. This, taken with the absence of this protein from PWYS3 cells identifies the 135K protein recognised by anti-Kex2C as Kex2p.

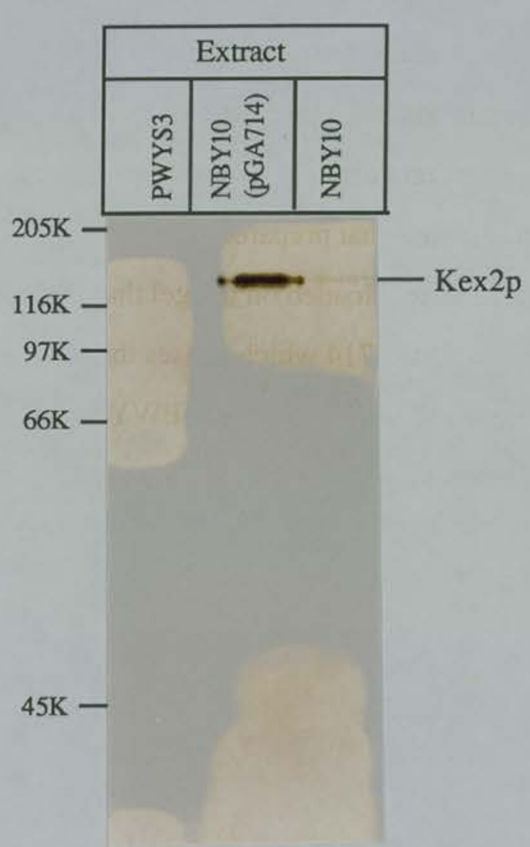

Extract
PWYS3
NBY10 (pGA714)
NBY10
205K
116K
97K
66K
45K
Kex2p

Figure 4-2.

Binding of Kex2 protein by a Kex2-Immunoadsorbent

Kex2-ImAd was prepared using 10μl anti-Kex2C (50μg protein) and 10μl Pansorbin (10% w/v *S. aureus* cell suspension). This was resuspended in 300μl of cell lysate (equivalent to 30 OD units of NBY10(pGA714) cells) and incubated on a rotating wheel, to allow gentle mixing, for 3 hours. The ImAd was then recovered by centrifugation (2 mins at 4000g) and washed 3 times (twice briefly by simple resuspension and once for 15mins on a rotating wheel) in lysis buffer. All steps were carried out at 4°C. The material thus recovered (ImAd bound) was resuspended either in 50μl Kex2 assay mix in order to assay the amount of Kex2 activity present (Figure 4-2a), or in SDS sample buffer to allow immunoblot analysis using anti-Kex2C as primary antibody following electrophoretic separation on a 10% polyacrylamide gel (Figure 4-2b). In both cases an aliquot of the yeast cell lysate (30μl, equivalent to 10% of that presented to the ImAd) was treated in the same way as the bound fraction (lysate). A control experiment was carried out in which 10μl Pansorbin to which no antibody had been bound was used in place of the ImAd (control bound).

N.B. Prior to the analysis of bound material by electrophoretic separation the samples were boiled for 5mins before the *S. aureus* cells were removed by centrifugation. The smear of immuno reactivity on the immunoblot in lanes containing samples of bound material is due to the release of protein A from the bacterial cells during this boiling.

Figure 4-2a

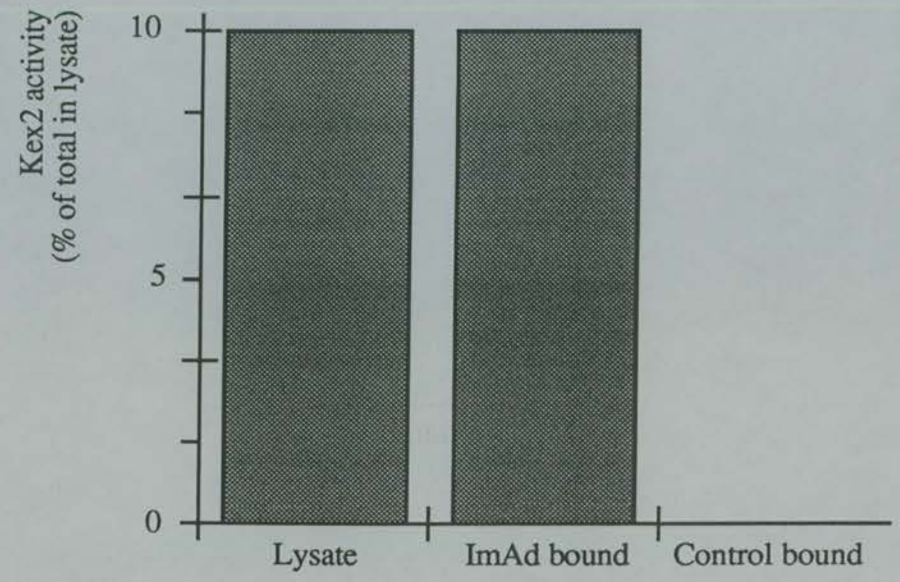


Figure 4-2b

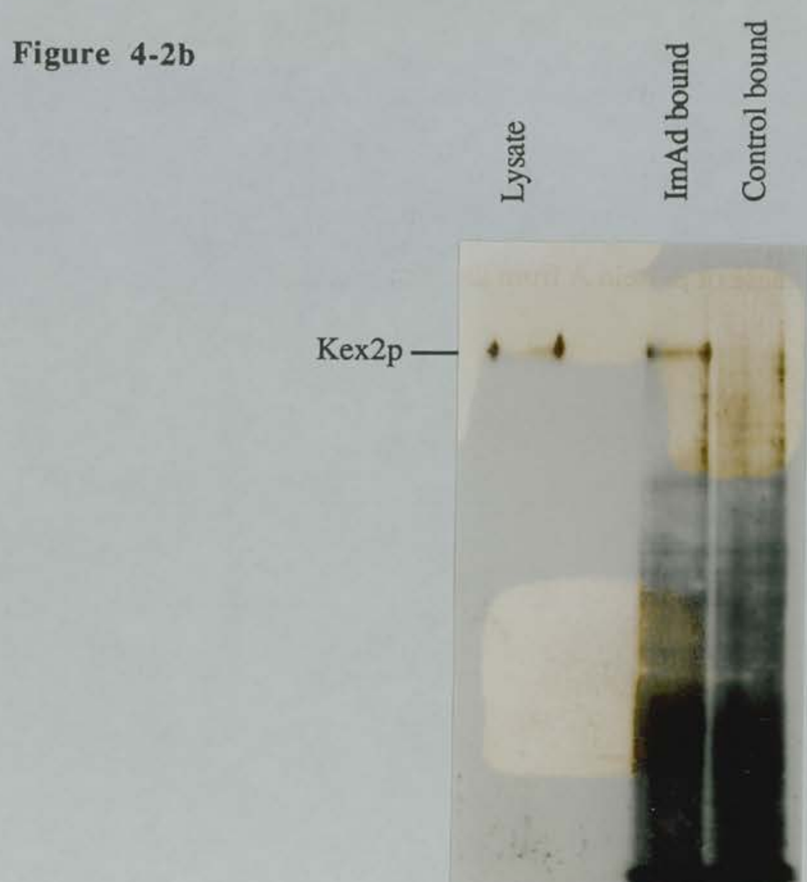
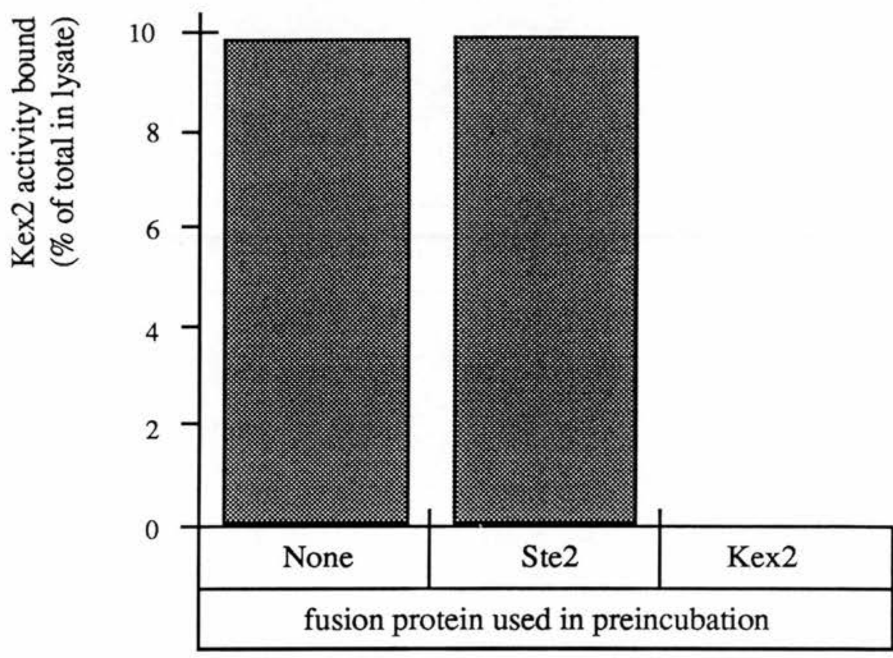


Figure 4-3.

Inhibition of Kex2p recovery by βgal-Kex2p

The procedure described in Figure 4-2 was used to recover Kex2p from an NBY10(pGA714) cell lysate using Kex2-ImAd that had been treated in one of three ways; prior to addition of the cell lysate the ImAd was incubated for 30 mins with a solution of one of two bacterially-produced βgal fusion proteins (βgal-Kex2C or βgal-Ste2p; in both cases 500μl of a 1mg/ml solution was used), or with PBS (control). The amount of Kex2 activity bound by the different preparations was assayed and expressed as a percentage of the total activity present in the lysate.


Kex2 activity bound
(% of total in lysate)
10
8
6
4
2
0
None
Ste2
Kex2
fusion protein used in preincubation

4.3. Optimisation of the recovery of Kex2p by immunoisolation

Under the conditions used for the preliminary experiments described above approximately 10% of the total Kex2 activity present in the lysate was bound by the ImAd (Figure 4-2). In an attempt to increase the recovery of Kex2p increasing amounts of anti-Kex2C were bound to Pansorbin in the formation of ImAd. From Figure 4-4 it can be seen that increasing the number of antigen binding sites in the ImAd above that achieved using 10μl (50μg) anti-Kex2C antibody appears to have no effect on the yield of Kex2p. In order to rule out the most obvious explanation for this result (that is, that not all of the antibody had bound to the Pansorbin, even though the amount added was within its binding capacity of 2mg/ml), it was confirmed that in each case all of the antibody added to the Pansorbin had indeed been incorporated into ImAd by showing that the supernatant remaining after the formation of the ImAd contained no anti-Kex2C (using immunoblot analysis; data not shown). Two other possible explanations for the observed result were considered. Firstly, it may be that only 10% of the Kex2 activity detectable in the lysate is capable of binding to the antibody, with the remaining 90% being in an inaccessible form. Alternatively, since it is expected that the antibody-antigen interaction in these experiments is resulting in the recovery of membrane vesicles containing the transmembrane Kex2 protein, it may be that these vesicles are of such a size that once a certain number (containing approximately 10% of total Kex2 activity) have bound to the ImAd, the *S. aureus* cells become completely coated with vesicles leaving no room for any more to bind even though there may be unfilled antigen binding sites.

It was shown that if Kex2-ImAd was used to recover Kex2 protease activity equivalent to 10% of the total from a cell lysate, and then that same, now depleted, lysate was exposed to an identical ImAd, then the second aliquot of ImAd also bound Kex2 activity (again equivalent to approximately 10% of the original total activity of the lysate), leaving a supernatant with a Kex2 activity equivalent to 80% of that assayed in the original crude lysate (Figure 4-5). This procedure could be

repeated until about 80% of the original Kex2 activity had been removed from the lysate demonstrating that about 80% of the Kex2 activity in a crude lysate is able to bind to ImAd, and thus ruling out the first explanation for the limitation upon Kex2 yield.

In order to test the second explanation given above, that the surface area of the ImAd is the factor limiting the amount of Kex2 protein recovered, the amount of Pansorbin used in the formation of the ImAd was increased. Figure 4-6a shows that by increasing the amount of Pansorbin used in ImAd formation in order to increase the total surface area of the ImAd (as well as increasing the amount of anti-Kex2C used, rather than binding a fixed amount of antibody to increasing amounts of Pansorbin, in case the amount of antibody also became a limiting factor in the amount of Kex2p recovered), it is possible to recover more than 10% of the activity present in a lysate in one round of binding, with an apparent maximum of 14% being recovered here. Suprisingly, increasing the surface area of the ImAd further (along with a concominant increase in the number of antigen binding sites) did not seem to lead to a further increase the amount of Kex2 activity recovered, but seemed to have the opposite effect (Figure 4-6a-bound). This observation did not agree with the results obtained from immunoblot analysis of the same material (Figure 4-6b) which indicate that the use of an increasing amount of ImAd leads to an increasing amount of Kex2p to be recovered from a lysate. The amount of activity left in the lysate following treatment with ImAd was assayed and when this data was analysed a discrepancy was found in that when these results were added to the amount of Kex2 activity assayed attached to the ImAd a total of less than 100% was obtained (i.e. activity recovered from, and activity left in, the lysate did not add up to 100%). The assays carried out on the supernatants remaining after treatment of lysates with ImAd show that up to 80% of Kex2 activity is removable from these lysates in a single round of binding (Figure 4-6a-unbound). Thus it appears that the presence of Pansorbin interferes with the Kex2 protease activity assay that was used in this study (Figure 4-7).

Figure 4-4.

An apparent maximum amount of Kex2p in a cell lysate can bind to Kex2-ImAd

A number of different Kex2-ImAd preparations made using 10µl Pansorbin and various amounts of anti-Kex2C (from 0-100µl; 0-500µg protein). These preparations were individually mixed with, and subsequently recovered from, NBY10(pGA714) cell lysates as described in Figure 4-2. The amount of Kex2 protease activity bound was assayed, expressed as a percentage of the total Kex2 activity present in the lysate, and plotted against the amount of anti-Kex2C used to prepare the ImAd.

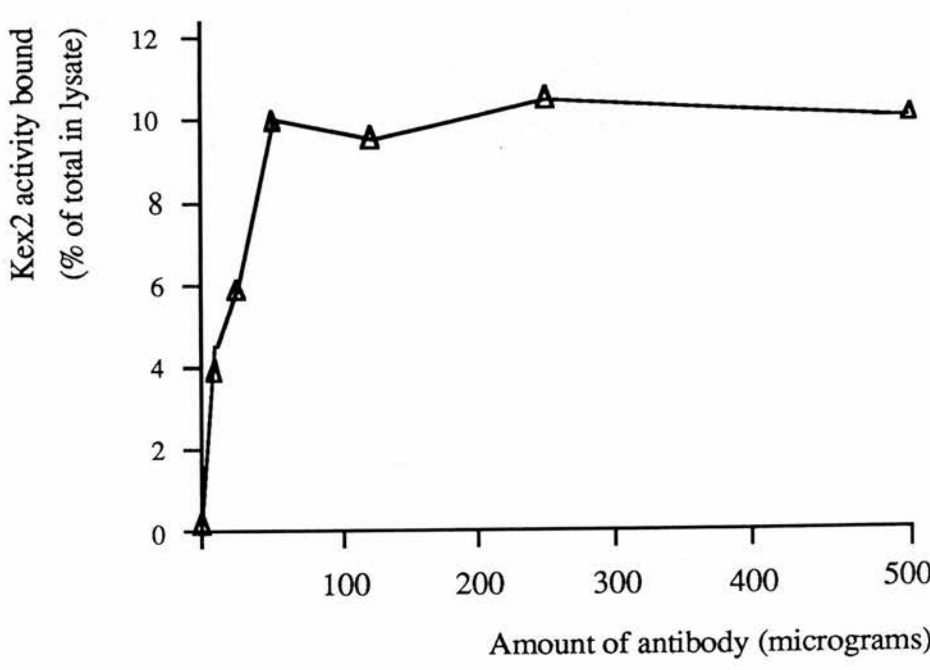
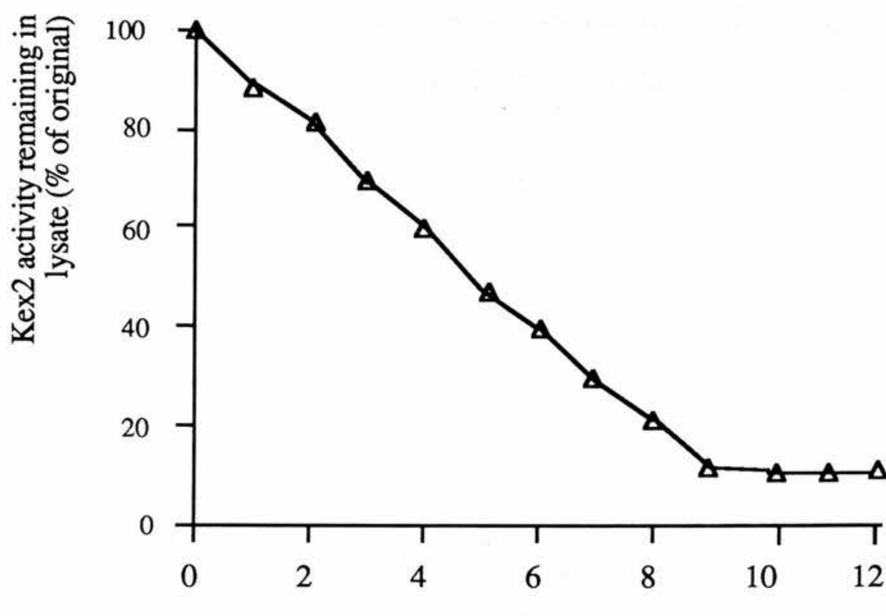

Kex2 activity bound
(% of total in lysate)
12
10
8
6
4
2
0
100
200
300
400
500
Amount of antibody (micrograms)

Figure 4-5.

Most of the Kex2 activity in a cell lysate can be bound by Kex2-ImAd

ImAd prepared using 10μl Pansorbin and 10μl anti-Kex2C (50μg protein) was used to recover 30 units of Kex2 activity from an NBY10(pGA714) cell lysate, leaving 270 units of activity in the supernatant. This depleted lysate was then exposed to an identical second aliquot of ImAd. The amount of Kex2 activity recovered by this was assayed, as was that remaining in the lysate. The lysate was exposed to further rounds of binding using fresh ImAd until the addition of such an ImAd led to no recovery of Kex2 activity and no further depletion of Kex2 activity from the lysate.



Kex2 activity remaining in lysate (% of original)
100
80
60
40
20
0
0
2
4
6
8
10
12
Number of rounds of exposure to ImAd

Figure 4-6.

Optimisation of Kex2p recovery

ImAd prepared using 500μl Pansorbin and 500μl anti-Kex2C (2.5mg protein) was divided into 7 portions (150, 100, 50, 25, 10, 5 and 2μl), each of which was used to bind Kex2 protein from an NBY10(pGA714) lysate as described in Figure 4-2. Kex2 activity bound by each portion of the ImAd was assayed (Figure 4-6a, ImAd bound), as was that remaining in the lysate following the removal of the ImAd (Figure 4-6a, ImAd unbound). The same procedure was carried out using an equivalent amount of Pansorbin to which no antibody had been bound in place of ImAd. This set of control experiments demonstrated that up to 150μl of Pansorbin alone, in place of ImAd, led to no recovery of Kex2 activity and no depletion of activity from the supernatant (data not shown).

Identical samples were prepared in parallel and processed for immunoblot analysis using anti-Kex2C as primary antibody as described in Figure 4-2 (Figures 4-6bi and 4-6bii). Two exposures of the immunoblot are shown in order to highlight the different amounts of Kex2 recovered from identical lysates by different amounts of ImAd. No detectable Kex2p was present in the material bound using Pansorbin alone in place of ImAd (data not shown).

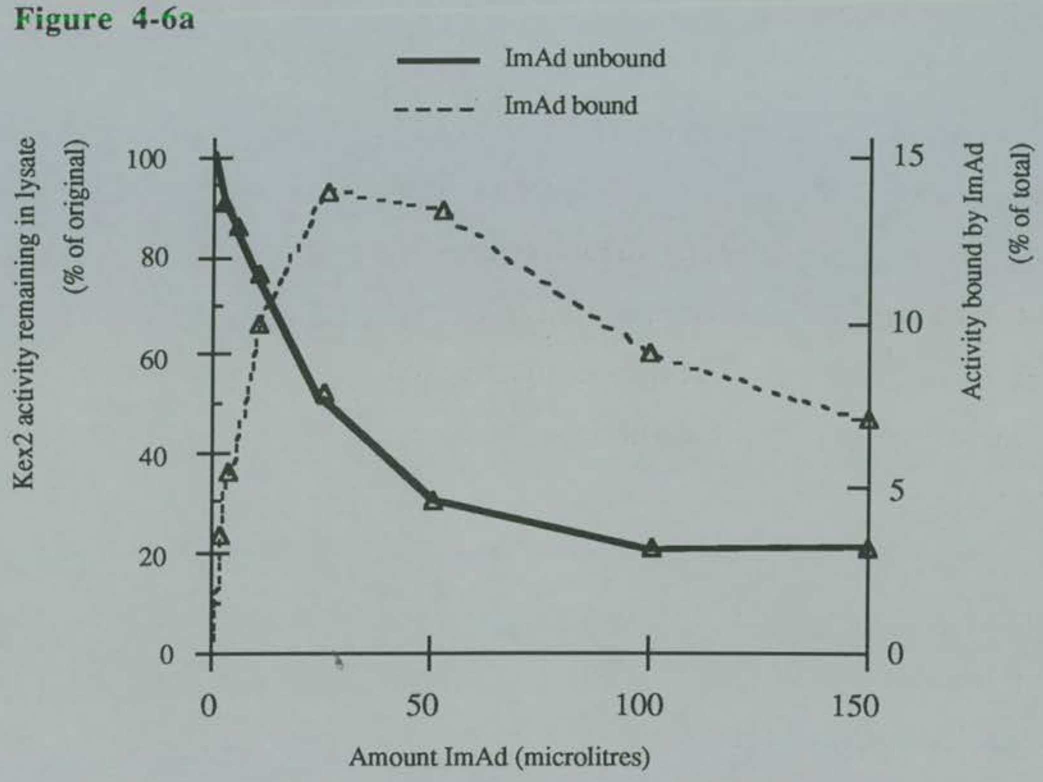


Figure 4-6a
ImAd unbound
ImAd bound
Kex2 activity remaining in lysate
(% of original)
100
80
60
40
20
0
15
10
5
0
Activity bound by ImAd
(% of total)
0
50
100
150
Amount ImAd (microlitres)

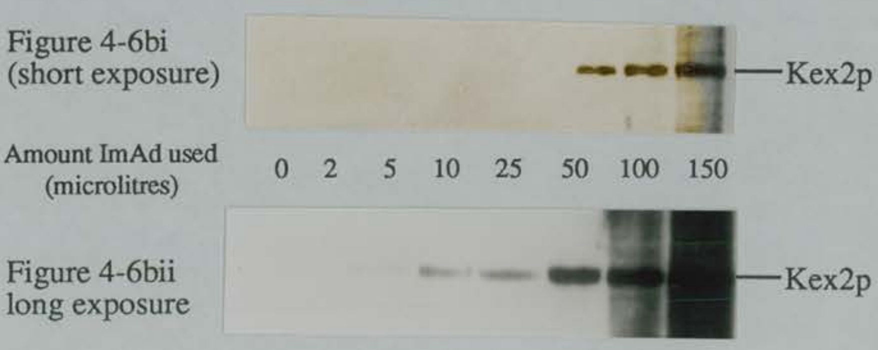


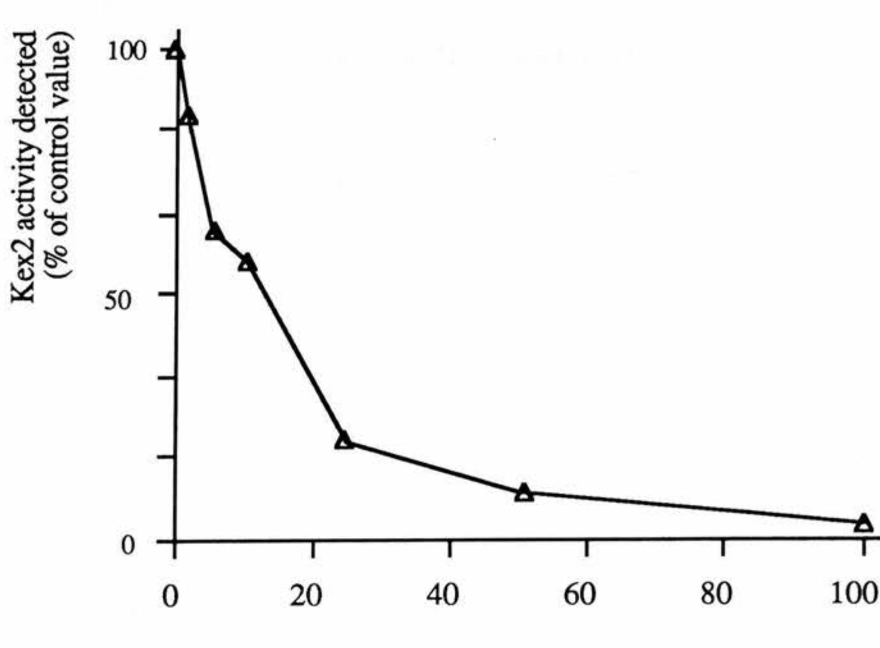
Figure 4-6bi
(short exposure)
Kex2p
Amount ImAd used
(microlitres)
0 2 5 10 25 50 100 150
Figure 4-6bii
long exposure
Kex2p

Figure 4-7.

Pansorbin inhibits the Kex2 assay

The amount of Kex2 activity in 30µl of an NBY10(pGA714) cell lysate (equivalent to 3 OD units) was assayed in the absence of, and in the presence of increasing amounts of Pansorbin. 2, 5, 10, 25, 50 and 100µl of Pansorbin were washed with lysis buffer before being resuspended in 30µl NBY10(pGA714) lysate. The amount of Kex2 protease activity present in these samples was assayed, as was that present in 30µl of lysate that had not been exposed to Pansorbin (control value).

Equivalent amounts of Pansorbin were resuspended in 30µl lysis buffer. No Kex2 activity was detected in these samples.


Kex2 activity detected
(% of control value)
100
50
0
0
20
40
60
80
100
Amount Pansorbin present in sample
(microlitres)

If the assumption is made that the activity removed from the lysates has been bound by the ImAd then it can be seen from Figure 4-6a that 80% of assayable Kex2 activity in an NBY10(pGA714) cell lysate can be removed using Kex2-ImAd. This result agrees with data presented in Figure 4-5 and therefore it can be concluded that 80% of the Kex2 activity detected by the assay used here in a NBY10(pGA714) cell lysate is in a form that can be recovered using Kex2C-ImAd.

From Figure 4-6 it can be seen that at least 100μl of a now standard Kex2-ImAd (prepared using 1ml (5mg) anti-Kex2C per ml Pansorbin (10% w/v *S.aureus* cell suspension)) is required to recover 80% of the Kex2 protease activity detectable in an NBY10(pGA714) cell lysate which seems to be the maximum amount of activity that can be removed from such a sample by the procedure used here (Figure 4-5). For all binding experiments reported so far, ImAd and cell lysates were incubated together for three hours prior to recovery of the ImAd for examination of material bound to it. Since it is hoped that material bound by Kex2-ImAd will be used to reconstitute protein transport, it was thought to be desirable to determine the shortest period of time with which the ImAd can be incubated with a cell lysate and recover Kex2p. Figure 4-8 demonstrates that it is necessary to incubate the ImAd with the cell lysate for at least 150 minutes in order to bind 80% of the Kex2 protein present in the lysate.

On the basis of the results presented in this chapter it was decided that in subsequent experiments where a fraction bound from a yeast cell lysate by Kex2-ImAd was required, the material which would be used would be that bound by 100μl ImAd (prepared using 1μl anti-Kex2C antibody (1μg protein) per μl (10% w/v *S.aureus* cell suspension) Pansorbin) from 300μl yeast cell lysate (equivalent to 30 OD units of yeast cells taken from a growing culture of OD_{600}=0.5-1.0).

Having optimised the conditions for binding Kex2p from an NBY10(pGA714) lysate, it was decided to investigate the possibility of recovering Kex2p from lysates prepared from cells expressing the protein at wild type levels. It was found to be necessary to use 100µl Kex2C-ImAd (prepared as described above) to remove all the Kex2p (detectable by immunoblot analysis) from 300µl of an NBY10 lysate. Figure 4-9 demonstrates that using half of this amount of ImAd results in incomplete removal of Kex2p from the lysate.

Figure 4-8.

Time course of Kex2p binding

ImAd prepared using 1ml Pansorbin and 1ml anti-Kex2C (5mg protein) was divided into 10 identical aliquots, each of which was resuspended in 300μl NBY10(pGA714) lysate (equivalent to 30 OD units). These samples were incubated on a rotating wheel at 4°C for various times after which the ImAd was recovered by centrifugation and the amount of Kex2 protease activity remaining in the lysate was assayed.

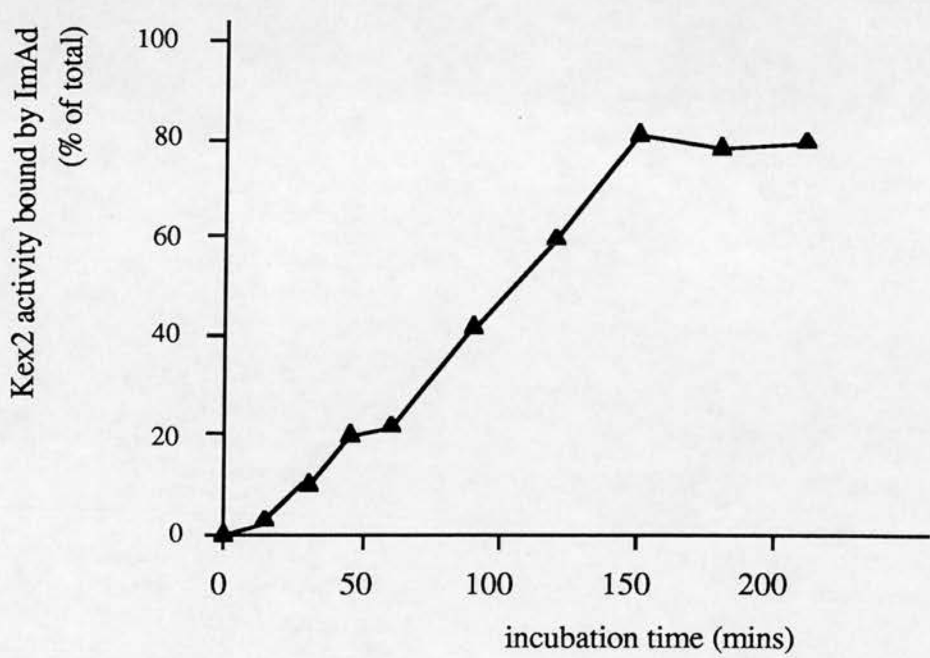
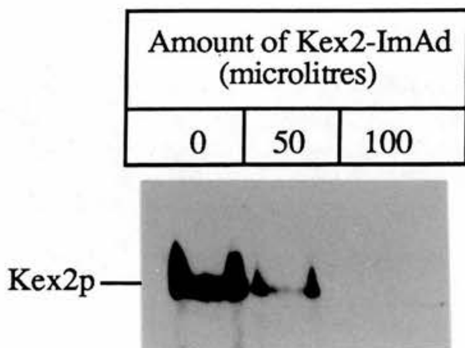

Kex2 activity bound by ImAd (% of total)
100
80
60
40
20
0
0
50
100
150
200
incubation time (mins)

Figure 4-9.

Binding of Kex2p from a lysate containing normal levels of the protein

100µl Kex2-ImAd (prepared using 5µl anti-Kex2C antibody (5µg protein) per µl (10% w/v *S.aureus* cell suspension) Pansorbin) was reuspended in 300µl of an NBY10 glass bead lysate (prepared from 30 OD units of yeast cells taken from a growing culture of OD_{600}=0.5-1.0). The ImAd was removed after 3 hours incubation at 4^oC on a rotating wheel and the remaining lysate was subjected to electrophoretic separation on a 10% polyacrylamide gel prior ro immunoblot analysis using anti-Kex2C as primary antibody. A similar experiment was performed using 50µl of the same ImAd.


Amount of Kex2-ImAd
(microlitres)
0
50
100
Kex2p

Chapter 5

Characterisation of material immunoisolated from yeast cells using an affinity-purified anti-Kex2p antibody

5.1. Introduction

This chapter describes the characterisation of material recovered from yeast cells by immunoisolation based on the affinity of anti-Kex2C for the C-terminal domain of Kex2p. It is demonstrated here that the material contains the Kex2 protease in intact membrane vesicles, and that the material is enriched for marker enzymes of the yeast Golgi but does not contain markers associated with the ER or the vacuole.

5.2. Enzyme activities present in ImAd-bound material

Based on experimental evidence (discussed in Chapter 1) that defines Kex2p as a resident membrane protein of a late compartment of the yeast Golgi the assumption was made that material recovered from yeast using Kex2-ImAd would be enriched in membranes derived from this organelle but not in those derived from other organelles such as the ER or the vacuole. It is envisaged that the most likely contaminating membranes in a preparation of Golgi membranes obtained using immunoisolation would be those derived from the ER since it will contain resident Golgi proteins, such as Kex2p, *en route* to their destination. To investigate the composition of the material bound by Kex2-ImAd from yeast cells, the activities of biochemical marker enzymes were assayed to screen for the presence of ER and vacuolar membranes (NADPH cytochrome c oxidoreductase activity was used as a marker for the presence of ER membranes (Schekman in Strathern *et al.*, 1982), and CPY activity (Schwencke *et al.*, 1983) and DPAP B activity (Bordallo *et al.*, 1984) were used as markers for the presence of vacuolar membranes). Figure 5-1a shows that material bound by Kex2-ImAd from a lysate prepared from the yeast strain NBY10 using glass bead breakage (containing 80% of the cellular Kex2 activity) contains negligible amounts of these enzymes (less than 0.5% of that present in the lysate presented to the ImAd).

The heat stable dipeptidyl aminopeptidase (DPAP A), encoded by *STE13* is responsible for the N-terminal maturation of α-factor following its cleavage by Kex2p and C-terminal maturation by Kex1p (Fuller *et al.*, 1988), and is commonly used as a marker

for the presence of yeast Golgi membranes. The ImAd bound material was found to contain little heat stable (Julius *et al.*, 1984) DPAP A activity (less than 2% of that present in the lysate presented to the ImAd), little more than was bound from the same cell lysate using Pansorbin alone. Although it has been suggested that DPAP A resides in a different cellular compartment from Kex2p (due to the presence of several Lys-Arg sites in the sequence predicted to form its catalytic domain; Fuller *et al.*, 1988), it has recently been reported that immunofluorescence studies produce a staining pattern similar to that observed for Kex2p (T. Stevens - quoted in Seeger and Payne, 1992). This, taken with the fact that the two enzymes act in the maturation of α-factor, suggests that Kex2p and DPAP A might reside in the same cellular compartment. Taking such evidence into account, it was perhaps suprising that the material bound by Kex2-ImAd, known to contain the vast majority of cellular Kex2p from a lysate, was not enriched in DPAP A activity. The lysate from which the ImAd-bound fraction had been recovered was prepared by vortexing yeast cells in the presence of glass beads. This method of lysis can be considered harsh, and is likely to cause considerable disruption to internal cellular structures. It was suspected that during glass bead lysate preparation, the Kex2p-compartment had become fragmented to such an extent that even if DPAP A and Kex2p do both reside in the same compartment, the occurrence of the two in the same vesicle (derived from the Kex2p-compartment) was rare.

In order to investigate this hypothesis a more gentle method of lysis was used to generate the cell extract from which a Kex2-ImAd bound fraction could be obtained, in the hope that less disruption to internal membranes would lead to the isolation of a fraction more representative of the Kex2p-compartment using Kex2-ImAd. Figure 5-2 demonstrates that a yeast cell lysate prepared by homogenisation of spheroplasts contains a higher proportion of a secretory protein in a cryptic form (i.e. requiring the addition of detergent for its detection) than a lysate prepared from the same cells using glass bead breakage. In a glass bead lysate prepared from the yeast strain JRY188(pYJS50) which secretes the bacterial enzyme β-lactamase only 30% of the detectable activity is present in a cryptic form . An extract prepared from the same cells

by homogenisation of spheroplasts in a hypertonic buffer contains over 90% of the enzyme in such a form (Figure 5-2) suggesting that the method of homogenisation described is less disruptive to internal membranes than glass bead lysis.

A Kex2-ImAd bound fraction was therefore recovered from yeast cells that had been lysed by homogenisation in hypertonic media following removal of their cell wall and was found to contain 25% of the DPAP A activity that had been in the homogenate (Figure 5-1b). The activity of both the vacuolar and the ER marker enzymes in this fraction was negligible. As well as providing evidence that Kex2p and DPAP A do reside in the same cellular compartment, these findings suggest the possibility that Kex2p and DPAP A reside in separate domains of that compartment. It is envisaged that these domains are physically separated by the harsh method of glass bead lysis whereas the more gentle lysis technique of homogenisation allows them to remain in the same membrane structure more often.

On the basis of these results, it was decided that all future Kex2-ImAd bound fractions should be prepared from cell extracts prepared by homogenisation, since it is considered that they are more likely to be representative of the organelle in which Kex2p resides in the cell.

Figure 5-1.

Enzyme activities associated with ImAd-bound material

The activities of various enzymes were measured in cell extracts, material recovered from these extracts using Kex2-ImAd, and the material remaining following treatment with ImAd. This figure compares the amount of activiy of each of these enzymes recovered from an NBY10 glass bead lysate using Kex2-ImAd (Figure 5-1a) with those recovered in the same way from a homogenate of the same cells (Figure 5-1b). The activities recovered by the ImAd are expressed as a percentage of the total activity found in the extract from which the bound fraction was obtained.

The same assays were performed on material recovered using Pansorbin alone, revealing that Pansorbin alone recovers negligible amounts of activities of any of the enzymes assayed here (data not shown).

Figure 5-1a

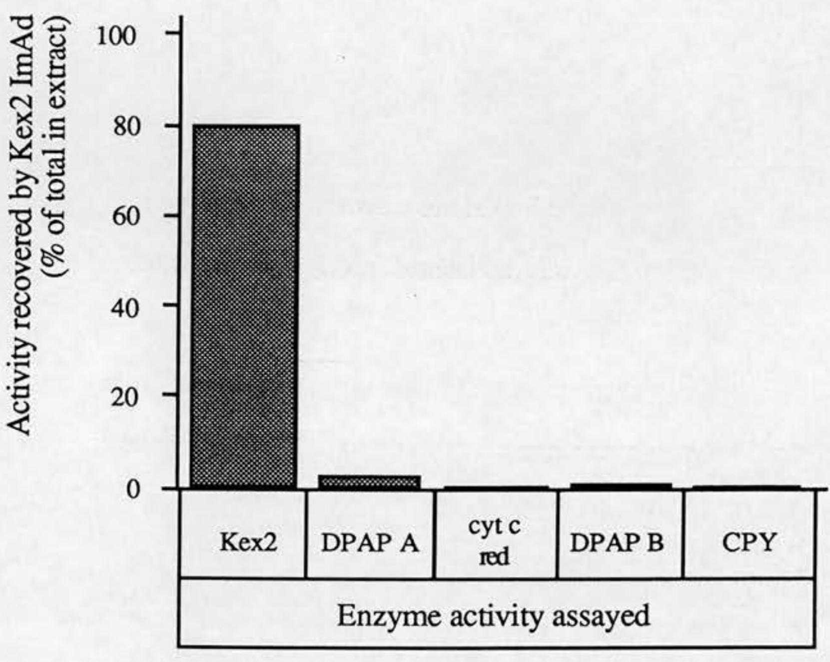


Figure 5-1b

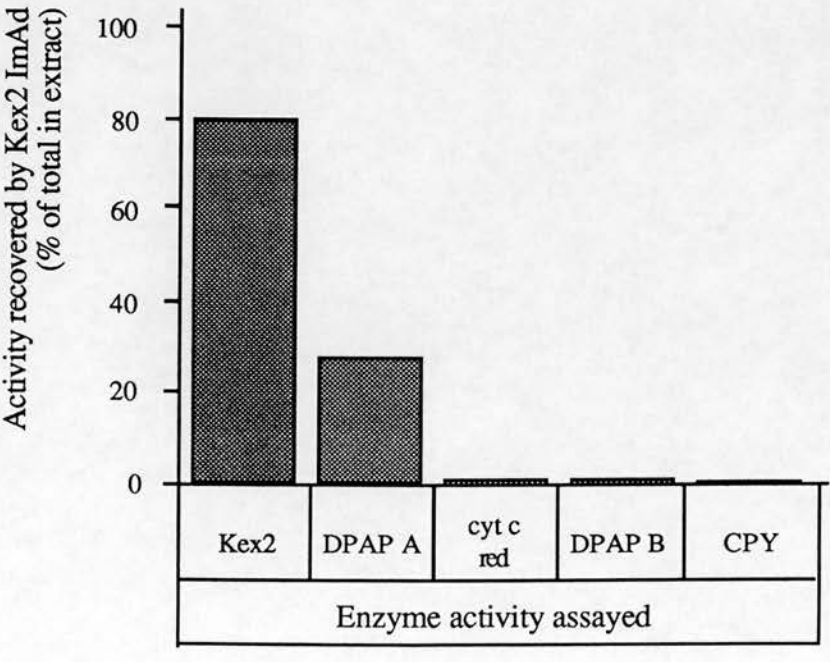
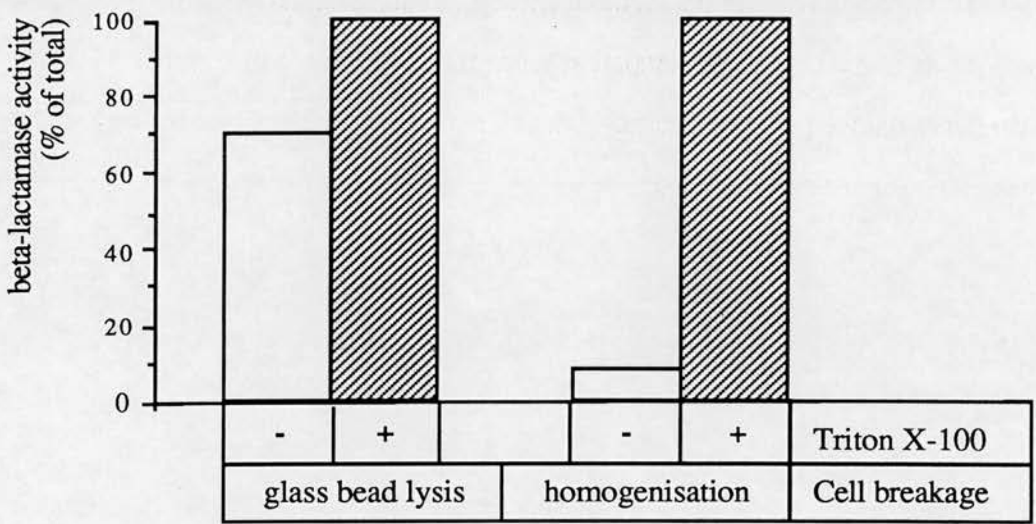


Figure 5-2

Glass bead lysis is more disruptive than homogenisation

Two extracts were prepared from the yeast strain JRY188(pYJS50) which actively secretes the bacterial enzyme β-lactamase. One extract was prepared using glass bead lysis and the other by homogenisation of spheroplasts. The activity of β-lactamase in 5μl of each extract (equivalent to 0.5 OD units of original culture) was assayed in 0.8M sorbitol both in the presence and absence of 0.1% Triton X-100 in order to determine what proportion of the enzyme was contained within membrane structures. The activity measured in the presence of detergent was taken as the total amount of activity present in the extract (the total amount of activity in the two extracts was found to be the same) and the activity measured in the absence of detergent was expressed as a percentage of this.


beta-lactamase activity
(% of total)
100
80
60
40
20
0
-
+
-
+
Triton X-100
glass bead lysis
homogenisation
Cell breakage

5.3. ImAd recovers Kex2p in sealed membrane vesicles

It is hoped that the material recovered by Kex2-ImAd from a yeast cell homogenate will be used to reproduce functions that are carried out in the cell, such as the fusion of transport vesicles with, and budding of transport vesicles from the Kex2p-containing compartment, and therefore it is important to establish whether it contains intact membrane vesicles derived from that organelle.

The ability of Kex2-ImAd to recover intact membrane vesicles from a yeast cell homogenate was initially demonstrated by the fact that it could be used to recover a soluble cargo protein from a homogenate prepared from cells that are actively secreting the protein. β-lactamase, a soluble protein secreted by JRY188 cells harbouring the plasmid pYJS50 can be recovered using Kex2-ImAd in such a form that its presence can only be detected under conditions associated with membrane disruption (Figure 5-3).

To demonstrate that the Kex2 protein recovered by Kex2-ImAd is itself contained within membrane vesicles, the susceptibility of the protein to externally added protease was examined. An affinity-purified antibody that recognises the N-terminal 120 amino acids of Kex2p (anti-Kex2N; produced by P. Whitley: Whitley, 1990) was used to this end. Intact vesicles bound to ImAd through the C-terminal domain of Kex2p will carry the region of the transmembrane protein recognised by anti-Kex2N inside them. Figure 5-4 shows that when material bound from a yeast cell homogenate by Kex2-ImAd is treated with protease prior to immunoblot analysis using anti-Kex2N as primary antibody, the protein detected is of an apparent molecular weight consistent with the cytoplasmic domain of Kex2p having been removed from the protein (approximately 100K; Fuller *et al.*, 1989a), whereas the same antibody recognises the full size protein in the same material prior to protease treatment. Protease treatment of ImAd-bound material carried out in the presence of detergent completely removes the protein recognised by anti-Kex2N from the sample.

Figure 5-3.

Recovery of intravesicular cargo protein using ImAd

An ImAd-bound fraction was prepared from a homogenate of JRY188 cells harbouring the plasmid pYJS50, as was a control bound fraction using Pansorbin alone. These cells secrete the bacterial enzyme β-lactamase. The two bound fractions were each split into two aliquots, one of which was resuspended in 50µl homogenisation buffer and the other in 50µl PBS containing 0.1% Triton X-100. ImAd was removed from each of these samples by centrifugation and the resultant supernatants were assayed for the presence of β-lactamase activity. The results are expressed as a percentage of the activity released from the ImAd-bound material by 0.1% Triton X-100.

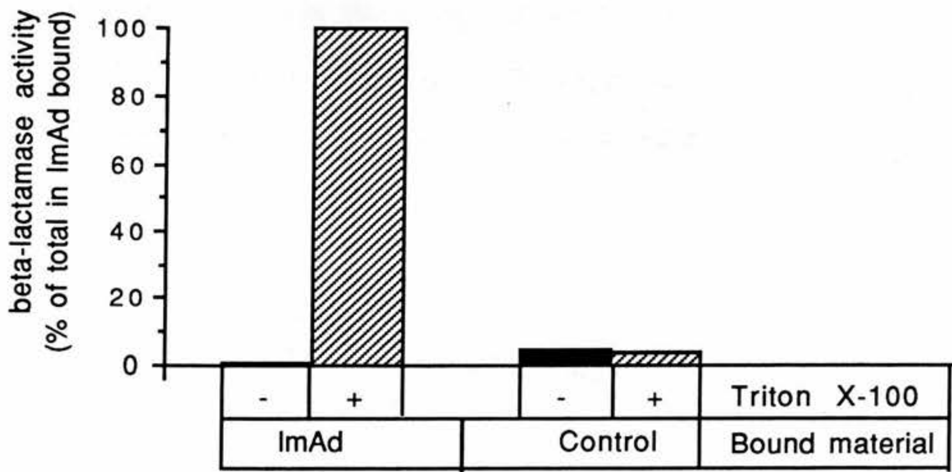

beta-lactamase activity
(% of total in ImAd bound)
100
80
60
40
20
0
-
+
-
+
Triton X-100
ImAd
Control
Bound material

Figure 5-4.

The luminal domain of immunoisolated Kex2p is inaccessible to proteinase K

Five aliquots of material bound by Kex2-ImAd from an NBY10 homogenate were resuspended in 50µl homogenisation buffer containing 2mM $CaCl_2$. Proteinase K was added to a final concentration of 50µg/ml as indicated (Triton X-100 was added to a final concentration of 0.1% prior to this where appropriate). The samples were placed on ice for 60 mins after which PMSF was added to a final concentration of 3mM. ImAd was removed from the samples after they had been boiled in SDS sample buffer for 5 mins. The samples were then subjected to 10% SDS-PAGE prior to transfer to nitrocellulose to allow immunoblot analysis using an affinity purified antibody which recognises the luminal, N-terminal domain of Kex2p. Included on the gel was a sample of the NBY10 homogenate, to ascertain the mobility of the full length Kex2 protein.

lysate	Treatment of Kex2-ImAd bound material				
	-	-	+	+	Proteinase K
	-	+	-	+	Triton X-100

Full length Kex2p

5.4. Composition of ImAd bound material

5.4a. Immunoblot analyses of the Kex2-ImAd bound fraction

The composition of the material bound by Kex2-ImAd was investigated in order to further characterise this fraction. This was done in two ways. Firstly, the material was tested for reaction with antibodies that had been raised against proteins known to reside in specific yeast organelles; and secondly, proteins recovered using ImAd that are not bound by Pansorbin were identified.

Immunoblot analyses of a Kex2-ImAd-bound fraction prepared from an NBY10 homogenate with antibodies prepared as described in the appendix to Chapter 3 were used to assess the composition of the fraction. The ImAd-bound material shows no reaction with the antiserum that was raised against Hmg1p even when extracted with detergent as described in the appendix to Chapter 3 (data not shown). This result is as expected, from the NADPH cytochrome *c* oxidoreductase assay results (see 5.2.) and provides additional evidence to suggest that the ImAd-bound fraction contains little, or no, contamination with membranes derived from the ER. Similarly, ImAd-bound material (prepared from an NBY10(pIH2-4) homogenate) does not demonstrate any reaction with the affinity-purified anti-Ste2p antibody (data not shown) which may be taken to suggest that the fraction does not contain any material derived from the plasma membrane. However, Figure 5-5a shows that a supernatant taken from the cell line B/1A1 (which recognises the βgal-Pma1p fusion protein (but not the βgal-Kex2p fusion protein), as well as a protein taken to be Pma1p in a P100 prepared from NBY10 - see the appendix to Chapter 3) reacts slightly with material bound by Kex2-ImAd from an NBY10 homogenate, but not with material bound from the same preparation by Pansorbin alone. This apparent contradiction over whether plasma membrane proteins can be detected in the material bound by the ImAd may be explained by the different abilities of the two antibodies used to detect their antigens in immunoblot analysis. To detect a signal relating to the recognition of Ste2p by the affinity-purified anti-Ste2p antibodies, it is necessary to expose x-ray film to a filter that

had been treated with ECL reagent (following immunoblot analysis) for 10 minutes, whereas, under the same conditions, a signal relating to the detection of Pma1p in a similar fraction by the B/1A1 supernatant can be detected after an exposure of approximately 10 seconds. An exposure of 2 hours was required to detect the reaction of B/1A1 supernatant with the ImAd bound material, demonstrating that the level of Pma1p contained within the fraction is very low. Although this may be taken to suggest that the ImAd bound fraction contains some membrane derived from the plasma membrane, the presence of Pma1p in the ImAd bound material can be explained as protein traffic travelling through the Kex2p-containing compartment *en route* to the plasma membrane.

The Kex1p carboxypeptidase acts in the processing of α-factor after Kex2p, but before DPAP A (Fuller *et al.*, 1988). Since the two enzymes which act immediately before and after Kex1p have been shown to reside in the same cellular compartment (see 5.2.), it seems highly likely that Kex1p will also reside in this compartment. To investigate this, an antibody that recognises Kex1p (supplied by Dr. H. Bussey, University of Montreal) was used in an immunoblot analysis of Kex2-ImAd bound material prepared from an NBY10 homogenate. Figure 5-5b shows that, as expected (H. Bussey - personal communication: Dmochowska *et al.*, 1987) the Kex1p antibody recognises a protein with an apparent molecular weight of approximately 60K in an NBY10 homogenate which is present in an ImAd-bound fraction prepared from such a homogenate, but not in material bound from the same preparation by Pansorbin alone. This result provides direct evidence that the compartment in which Kex2p resides inside the cell, contains Kex1p, as well as DPAP A, and that the material bound from a yeast cell homogenate resembles the compartment in which Kex2p resides inside the cell.

Figure 5-5.

Immunoblot analyses of the Kex2-ImAd bound material

Material was recovered from an NBY10 homogenate using both Kex2-ImAd and Pansorbin alone. Both of these fractions (Kex2-ImAd bound and Pansorbin bound) were tested for reaction with supernatant from the cell line B/1A1 (Figure 5-5a), which recognises Pma1p, by immunoblot analysis, following electrophoretic separation on a 10% polyacrylamide gel. A similar analysis was performed on the same material using an anti-Kex1p antibody (supplied by Dr. H. Bussey) - Figure 5-5b. A P100 prepared from the same volume of NBY10 homogenate from which the ImAd bound fraction had been prepared was also included in this analysis (P100).

Figure 5-5a

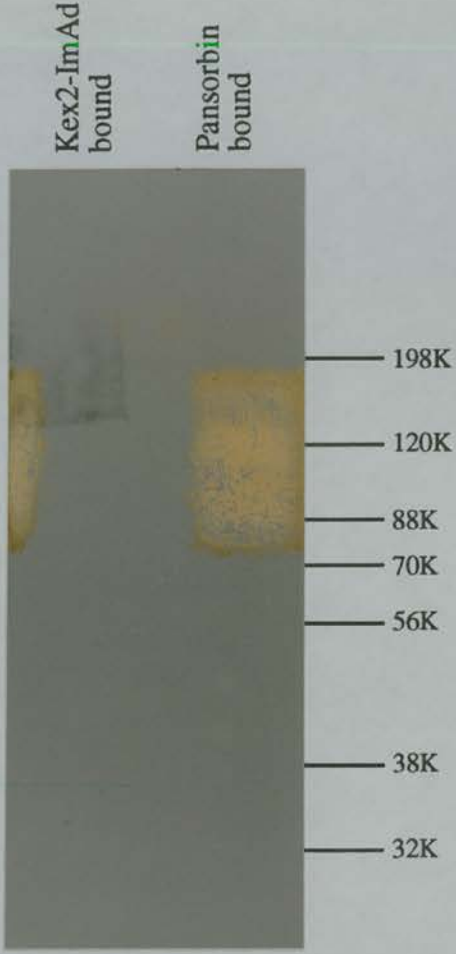
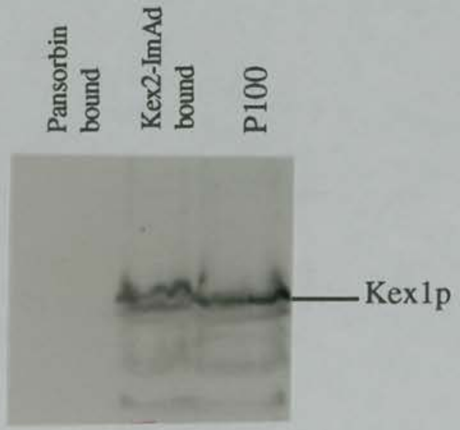


Figure 5-5b



5.4b. Polypeptide composition of Kex2-ImAd bound material

Kex2-ImAd was used to recover a bound fraction from a homogenate of NBY10 cells containing proteins that had been radiolabelled using ^{35}S-methionine. This material was compared to that bound by Pansorbin alone. It can be seen from Figure 5-6 that the Kex2-ImAd recovers a number of radiolabelled proteins from an NBY10 homogenate that are not recovered from the same sample by Pansorbin alone. In an attempt to accentuate the differences between the fraction bound by ImAd and that bound by Pansorbin alone, the two bound fractions were prepared from a radiolabelled NBY10 homogenate that had previously been cleared using Pansorbin. It was hoped that this would lead to no, or little, material binding to Pansorbin in the second round of binding, but as can be seen from Figure 5-6, this does not appear to be the case. This may be due to the fact that the binding experiments are carried out over a long period of time (3 hours), during which proteins in the homogenate may denature, such proteins may tend to form aggregates, which would sediment during recovery of the bound fraction. Although this attempt to clean-up the control bound fraction has been only partly successful, it can be seen that some proteins that are unique to the ImAd bound fraction prepared from the uncleared homogenate are also found in the ImAd bound fraction prepared from the cleared homogenate (but not in the fraction bound by Pansorbin alone from either sample). Five proteins that are present in both of the ImAd bound fractions, but in neither of the control bound fractions are indicated on Figure 5-6. It is likely that these proteins are resident to the same compartment as Kex2p, and their characterisation would provide further insight into the function(s) of this organelle.

It is worth noting that no band on the autoradiogram relating to Kex2p has been identified specifically to material bound by Kex2-ImAd, even though this material is known to contain the protein. This may demonstrate some technical limitation of using such an approach to identify proteins recovered using Kex2-ImAd.

Figure 5-6.

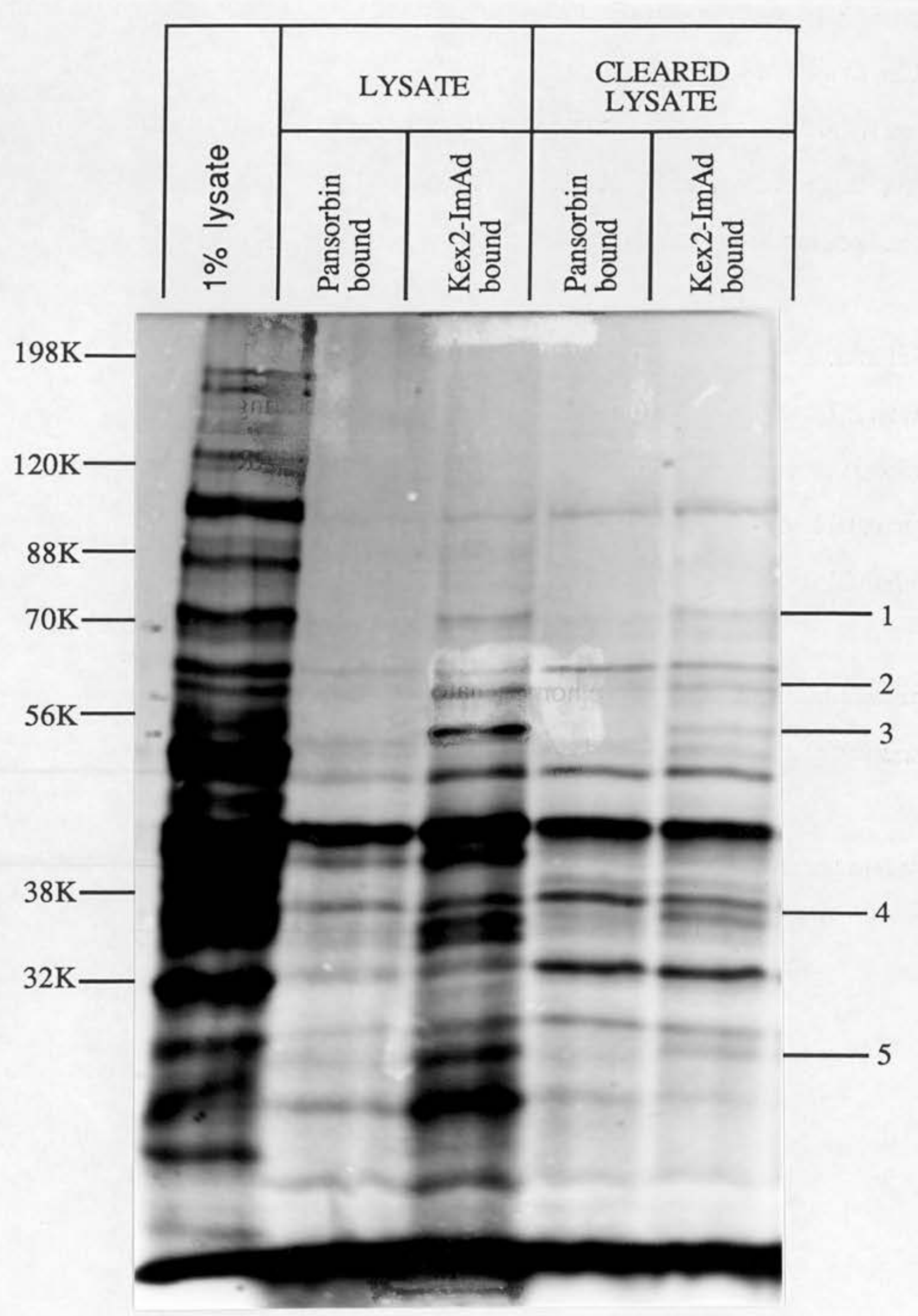
Identification of proteins specific to the Kex2-ImAd bound fraction

An ImAd bound fraction was prepared from an NBY10 homogenate containing proteins that had been radiolabelled using ^{35}S-methionine. This (Kex2-ImAd bound-LYSATE) was electrophoretically separated on a 10% polyacrylamide gel. Material recovered from the same homogenate using Pansorbin alone was processed on the same gel (Pansorbin bound - LYSATE). The gel was processed by fluorography, and the resultant autoradiogram is shown here.

An ImAd bound fraction was prepared from the same homogenate that had been exposed to 100μl Pansorbin for three hours at 4°C on a rotating wheel. This material was analysed as described above (Kex2-ImAd bound - CLEARED LYSATE), as was that recovered from the same sample using Pansorbin alone (Pansorbin bound - CLEARED LYSATE).

Included on the gel was 3μl of the homogenate (equivalent to 1% of that from which the bound fractions were prepared - 1% lysate).

Indicated on the figure are the positions of the five proteins discussed in the text.


1% lysate
LYSATE
CLEARED LYSATE
Pansorbin bound
Kex2-ImAd bound
Pansorbin bound
Kex2-ImAd bound
198K
120K
88K
70K
56K
38K
32K
1
2
3
4
5

5.5. Kex2-ImAd bound fraction can function as an acceptor compartment in a protein transport assay

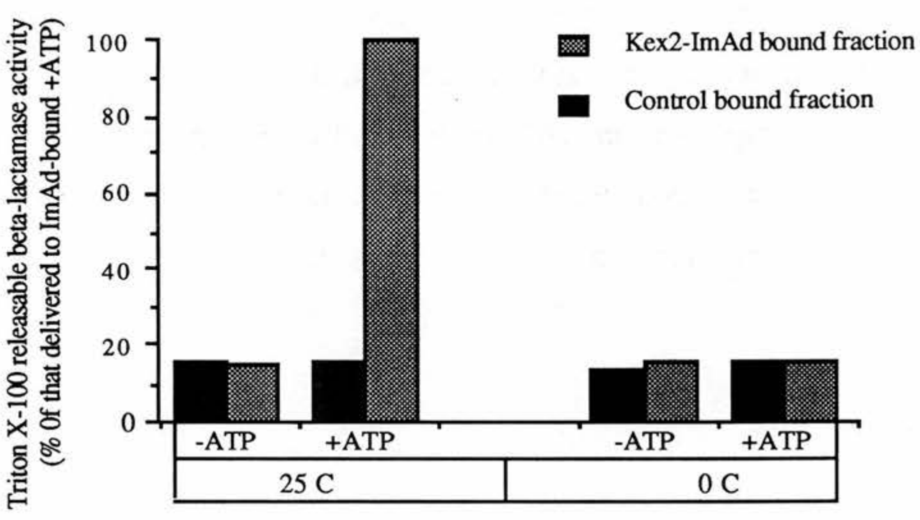
A yeast cell that is actively secreting a protein will contain that protein, in some form, in each compartment of its secretory pathway, and therefore theoretically, a lysate of such a strain can act as the donor fraction in a protein transport assay to follow delivery of the protein to any acceptor compartment (providing that the delivery can be assayed). A protein transport assay was devised using a Kex2-ImAd bound fraction prepared from NBY10 as the acceptor fraction and a cell homogenate prepared from yeast cells secreting the bacterial enzyme β-lactamase (JRY188(pYJS50)) as donor. After incubation of acceptor with donor under various conditions the ImAd-bound fraction was re-isolated and assayed for the presence of transported β-lactamase. It was found to be possible to detect the delivery of β-lactamase to Kex2-ImAd bound material, and the following conditions were found to be required for this delivery:

Figure 5-7 shows that delivery of β-lactamase from a donor fraction prepared from JRY188(pYJS50) cells that are secreting the enzyme, to an acceptor fraction, consisting of material recovered from an NBY10 homogenate (which contains no β-lactamase activity) requires energy in the form of an ATP-regenerating system. The delivery does not occur if the reaction mixture is incubated at 0°C instead of 25°C. It was also found necessary that the homogenate used as the donor fraction was more concentrated than the homogenate routinely used throughout this study (equivalent to 500 OD units/ml as oppose to 100 OD units/ml was required - data not shown)

Figure 5-7.

A protein transport assay following the delivery of a soluble cargo protein to Kex2-ImAd bound material

Material bound to either Kex2-ImAd, or Pansorbin alone, from an NBY10 homogenate, was reusupended in 90μl of a homogenate prepared from JRY188(pYJS50) on ice (the homogenate contained the equivalent of 500 OD units/ml). Either 10μl homogenisation buffer (-ATP), or 10μl of a 10 x ATP regenerating system (final concentration 1mM ATP, 8mM creatine phosphate, 30units/ml creatine phosphokinase: +ATP) was added, as indicated. These reaction mixtures were either left on ice (0°C), or incubated at 25°C for 15 mins, as indicated. After this time, the ImAd (or Pansorbin) was recovered by centrifugation, washed twice with homogenisation buffer, and then resuspended in PBS containing 0.05% Triton X-100. Material released from the ImAd (Pansorbin) by the detergent was assayed for β-lactamase activity. This is shown opposite, with the results expressed as a percentage of the highest value recorded.


Triton X-100 releasable beta-lactamase activity
(% Of that delivered to ImAd-bound +ATP)
100
80
60
40
20
0
Kex2-ImAd bound fraction
Control bound fraction
-ATP
+ATP
-ATP
+ATP
25 C
0 C

To dissect the transport being followed in the transport assay described above an experiment in which membranes prepared from NBY10 were added to the assay mixture was performed. It was found that the addition of these membranes caused a decrease in the amount of β-lactamase delivered to the ImAd bound fraction. If membranes prepared from an NBY10 homogenate that have been depleted of Kex2p-compartment by incubation with Kex2-ImAd are added to the assay mix, this competition effect was reduced. This supports the hypothesis that the assay measures the transport of cargo protein to the Kex2p-compartment, since an inhibition by the presence of membranes containing Kex2p-compartment is partially reduced by identical membranes which lack the Kex2p-compartment (Figure 5-8). One criticism of this experiment may be that the effect observed is merely due to the 'depleted membrane' fraction containing less material than the membranes which compete with the acceptor fraction in this assay. Such an explanation can be discounted, since it can be seen from Figure 5-6 that the ImAd bound material is made up of less than one percent of the total amount of protein found in a homogenate. The most generous of estimates might say that membrane proteins account for, say 10% of this, so at the very least the 'depleted membrane' fraction contains 90% of the amount of membrane material found in the membrane preparation which inhibits the assay (and probably more than this).

Figure 5-8.

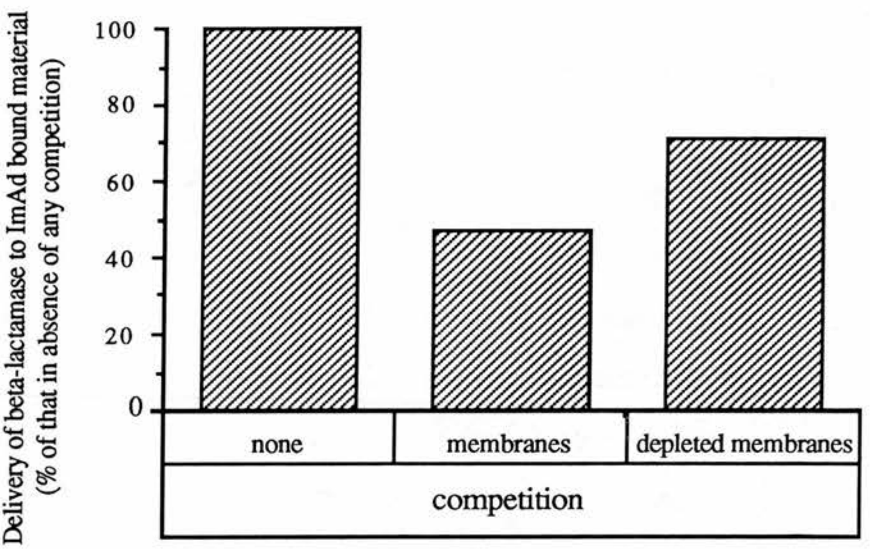
Kex2-ImAd partially depletes yeast membranes of the ability to compete with Kex2-ImAd bound material as an acceptor compartment in a protein transport assay

Material bound to Kex2-ImAd, from an NBY10 homogenate, was resuspended in 90µl of either a JRY188(pYJS50) homogenate (no competition), or an identical homogenate that had previously (immediately prior to resuspension of the ImAd-bound fraction) been used to resuspend either;

a P100 prepared from an NBY10 homogenate (membranes);

or a P100 prepared from an NBY10 homgenate that had been exposed to Kex2-ImAd for three hours, and now contained only 20% of its original Kex2p activity (depleted membranes).

10µl of an 10 x ATP regenerating system (described in Figure 5-7) was added to each of these reaction mixtures prior to incubation at 25°C for 15 mins. After this time the ImAd-bound fraction was recovered and treated as described in Figure 5-7. The results of the β-lactamase assays that were carried out in this experiment are expressed as a percentage of the activity delivered to an ImAd bound fraction by the JRY188(pYJS50) homogenate that had not had any membranes added to it.

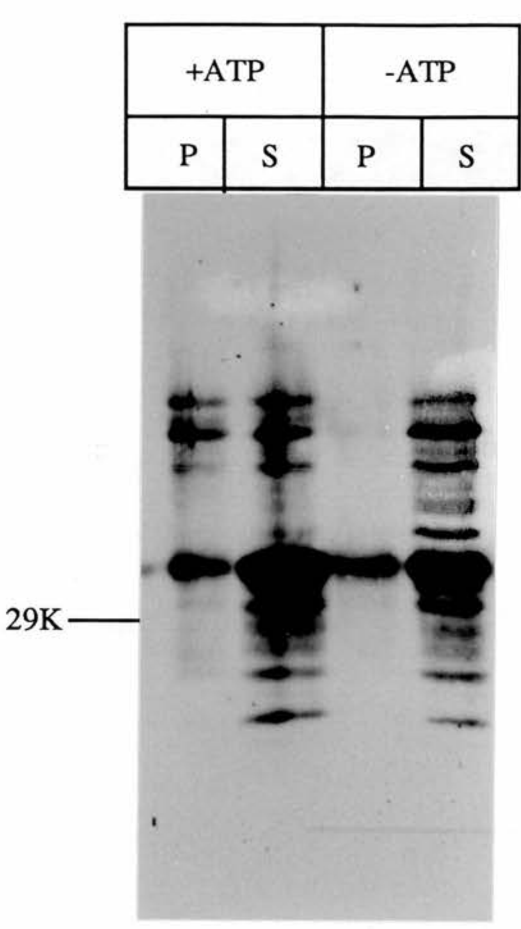

Delivery of beta-lactamase to ImAd bound material
(% of that in absence of any competition)
100
80
60
40
20
0
none
membranes
depleted membranes
competition

Delivery of proteins from a donor fraction to an acceptor fraction can be divided into two stages: Docking, whereby transport vesicles which have budded from the donor membrane attatch to, but do not fuse with, the acceptor compartment; and fusion, whereby the contents of the transport vesicles are delivered to the acceptor compartment, following a membrane fusion event. When a transport vesicle docks onto its acceptor membrane its contents remain physically separated from those of the acceptor compartment, whereas a membrane fusion event causes the contents of the two compartments to become mixed. To ascertain whether the assay described above follows the fusion of transport vesicles with the Kex2p-compartment bound to the ImAd, the form of the protein delivered to the bound fraction was examined. For this experiment, the donor fraction was prepared from PWYS3(pYJS50) cells (which lack Kex2p, and therefore cannot cleave the gene product of pYJS50). Delivery of the β-lactamase encoded by pYJS50 to a compartment containing Kex2p will result in a change in its electrophoretic mobility following cleavage by the peptidase. As can be seen from Figure 5-9 the area of the immunoblot where the expected Kex2p-cleaved product would be expected to be found (bacterially expressed β-lactamse was included on the immunoblot as a marker) demonstrates high reactivity with the anti-β-lactamase antibody used and the expected change in the electrophoretic mobility of the β-lactamase has not been detected. However, it can be seen that the material recovered from an assay carried out in the presence of an ATP regenerating system contains high molecular weight forms of β-lactamase that are not present in material recovered from an assay carried out in the absence of such an energy supply. Preliminary analysis of the different forms of β-lactamase detected in Figure 5-9 identify this material as being characteristic of that found in the Golgi (by comparison with an immunoblot shown in Graham and Emr, 1991) and the observation that the delivery of this material to the Kex2-ImAd bound fraction requires energy suggests that protein transport to the Kex2p-compartment is indeed being followed. Obviously, further work is required to determine the meaning of this result, but the preliminary work presented here provides strong evidence that material recovered from yeast cells by Kex2-ImAd, will be of use in the study of function(s) of the Kex2p-compartment.

Figure 5-9

Examination of β-lactamase delivered to ImAd bound material

ImAd bound material, prepared from an NBY10 homogenate, was resuspended in 90μl of a PWYS3(pYJS50) homogenate. 10μl of either homgenisation buffer, or a 10 x ATP regenerating system (described in Figure 5-7) was added prior to incubation at 25°C for 15 mins (+ATP/-ATP). The ImAd-bound material was recovered, washed three times in homogenisation buffer, and then boiled for 5 mins in SDS sample buffer. These samples (pellets; P on figure), as well as the supernatant (S on figure) remaining following the removal of the ImAd-bound material, were subjected to electrophoretic separation on a 10% polyacrylamide gel before being transferred to nitrocellulose to allow immunoblot analysis using an anti-β-lactamase antibody. Indicated on the figure is the position at which bacterially produced β-lactamase migrated to (29K), this is where Kex2p processed form of the protein expressed from pYJS50 would be expected to migrate to.


+ATP
-ATP
P
S
P
S
29K

Chapter 6

Immunoisolation of material from yeast using a Kex2-protein A hybrid protein

6.1. Introduction

Following the successful use of Kex2p in the immunoisolation of the compartment in which it resides (discussed in Chapters 4 and 5), it was decided that the protein should be used to investigate the use of the affinity of protein A for IgG to isolate specific membrane fractions from yeast using protein A fusion proteins. This was to be undertaken by constructing a gene fusion to encode a protein consisting of protein A IgG-binding domains fused onto the extreme C-terminus of Kex2p. It was envisaged that, if this fusion protein had the same subcellular localisation as Kex2p, it should be possible to use it to isolate the same membrane fraction that was isolated using Kex2-ImAd, by using the Fc portion of IgG molecules attached to a matrix. This possibility was investigated since, if such an approach was found to be successful, it could be adapted to isolate other membrane fractions (by choosing appropriate proteins with which to construct protein A fusion proteins). Such an approach may be advantageous over obtaining antibodies for the chosen proteins since the affinity of protein A for IgG is known to be high, whereas there is an element of chance involved in obtaining antibodies with a high affinity for a specific antigen.

6.2. SpaK; the Kex2p-protein A fusion protein

The oligonucleotides described in Figure 6-1 were used to generate a 3.6kb fragment of DNA, containing the entire coding region of the *KEX2* gene, by PCR using genomic DNA prepared from NBY10 as a template. The product from this reaction, which consists of DNA from 1200bp upstream of the *KEX2* gene (and is thought to contain the promoter region of the gene) was used to construct a *KEX2-spa* gene fusion, contained within the plasmid pNB66, as described in Figure 6-1. The gene fusion is predicted to encode a protein with a molecular weight of 107K (968 residues) consisting of 813 residues encoded by the *KEX2*-derived portion of the gene followed by 7 encoded by polylinker DNA and 148 residues encoded by DNA from the *spa* gene (as contained within pKpraSH; Figure 6-1). pNB66 was transformed into the yeast strain JRY188 to create JRY188(pNB66). Lysates from this strain were screened for

the presence of a protein A fusion protein by immunoblot analysis using a rabbit IgG-HRP conjugate. Figure 6-2 demonstrates that JRY188(pNB66) produces a protein recognised by rabbit IgG that has an apparent molecular weight of approximately 150K (no such protein is produced by JRY188). The predicted molecular weight of Kex2p is 90K but it has an apparent molecular weight of 135K due to the high net negative charge that it carries in its C-terminal domain (Fuller *et al.*, 1989). The portion of protein A fused to Kex2p in the construction of SpaK is predicted to add approximately 16K onto the molecular weight of Kex2p and therefore it is concluded that the protein detected in Figure 6-2 is the expected SpaK fusion protein encoded by the gene fusion harboured by pNB66. Figure 6-2 also shows that the fusion protein expressed by NBY10(pNB66) fractionates to the P100 prepared from a cell lysate, with none being found in the soluble S100. This result is consistent with the protein being associated with membranes.

To demonstrate that SpaK exhibits Kex2 protease activity pNB66 was transformed into a *kex2* mutant strain (PWYS3). Extract prepared from the transformant PWYS3(pNB66) was found to contain levels of Kex2 protease activity comparable to those found in a similar extract prepared from JRY188 cells whereas extract prepared from PWYS3 cells contains little of this activity (Figure 6-3).

The results presented so far in this chapter, that SpaK is produced by JRY188(pNB66) cells at a level comparable to that at which JRY188 cells produce Kex2p, and that the protein is associated with membranes, were as predicted, and therefore the investigation of the use of SpaK to recover the Kex2p-containing compartment was continued.

Figure 6-1.

Creation of the *KEX2-spa* gene fusion

The oligonucleotides 5'-GCGGATCCGTCGATCGTCCGGAAGATGG-3' and 5'-CGGGATCCGGTACCTCCAGTGCAACCAAACG-3' were used as primers for the PCR using genomic DNA prepared fron NBY10. This gave rise to a 3.6kb product which, following digestion with *Bam*HI and *Kpn*I, was cloned into similarly digested pK19 to create pNB64. The gene fusion was created by cloning a 0.5kb piece of DNA encoding IgG-binding domains of protein A from pKpraSH into pNB64 using *Sal*I and *Hin*dIII to create pNB65.

pKpraSH was created from pKpra using a two step strategy. A self-complementary linker oligonucleotide (5'-AGCTGGTCGACC-3') was cloned into the *Hin*dIII site of pKpra, destroying the *Hin*dII site and introducing a *Sal*I site, to create pKpraSB. The *Bam*HI site in pKpraSB was destroyed and a *Hin*dII site was introduced in its place by digesting the plasmid with *Eco*RI and *Bam*HI and inserting the oligonucleotides 5'-AATTGTAAGCTTG-3' and 5'-GATCCAAGCTTAC-3' into the digested plasmid to create pKpraSH.

pNB65 carries the *KEX2-spa* gene fusion flanked by *Bam*HI sites. The gene fusion was inserted into the *Bam*HI site of YCplac22 (in the orientation which gives rise to its expression from the *lacZ* promoter) to create pNB66.

Figure 6-2.

SpaK; the Kex2p-protein A fusion protein

Glass bead extracts were prepared from 100ml cultures of JRY188 and JRY188(pNB66) that had been grown in minimal media to an OD_{600} of 0.5. The extract prepared from JRY188(pNB66) was fractionated by centrifugation at 100 000g for 1 hour giving rise to a crude preparation of membranes (P100) and a soluble fraction (S100). The samples were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose prior to immunoblot analysis using a rabbit-HRP conjugate.

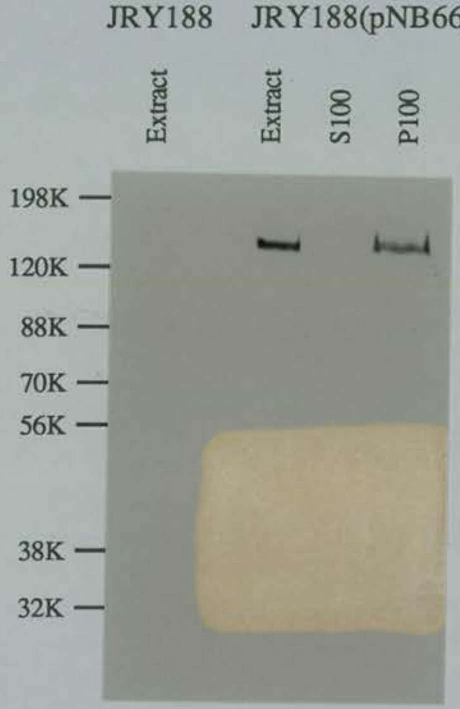
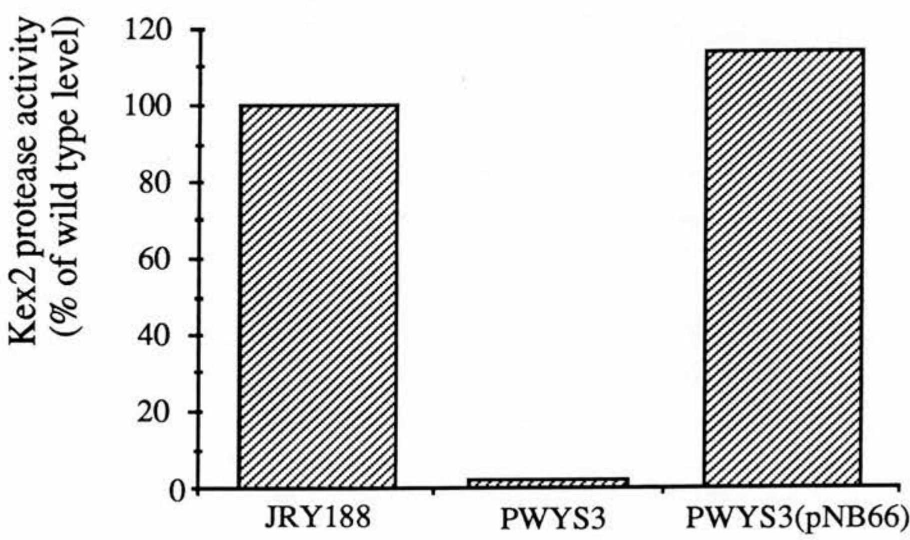

JRY188
JRY188(pNB66)
Extract
Extract
S100
P100
198K
120K
88K
70K
56K
38K
32K

Figure 6-3.

SpaK has Kex2 protease activity

pNB66 was transformed into the *kex2* mutant strain PWYS3. Glass bead extract was prepared from these cells, PWYS3(pNB66), as well as from untransformed PWYS3 and JRY188. The level of Kex2 protease activity was assayed in each of these extracts and was expressed as a percentage of the level found in JRY188.


Kex2 protease activity
(% of wild type level)
120
100
80
60
40
20
0
JRY188
PWYS3
PWYS3(pNB66)

6.3. Recovery of SpaK-containing membranes

Using the protocol established for the recovery of Kex2p-containing membranes (Chapters 4 and 5) IgG-Sepharose was used in place of Kex2C-ImAd in an attempt to recover SpaK-containing membranes from an JRY188(pNB66) homogenate. Suprisingly, it was found that no SpaK was recovered from such a preparation using IgG-Sepharose (data not shown). Subsequent analysis of the homogenate revealed that the fusion protein was found in the pellet resulting from the low speed centrifugation that is performed following homogenisation of the cells (Figure 6-4). Kex2p is found in the supernatant that results following this centrifugation (Figure 6-4). This result suggests that SpaK does not reside in the same cellular compartment as Kex2p.

The possibility that SpaK is localised to the vacuole was considered following two recent reports which suggest that the C-terminal tail of Kex2p is important for its retention in its compartment and that the vacuole may be the default compartment for membrane proteins in yeast (Roberts *et al.*, 1992: Seeger and Payne, 1992). In order to investigate this, IgG-Sepharose was used to recover SpaK-containing membranes from a glass bead lysate prepared from JRY188(pNB66). The fusion protein did not appear in the pellet obtained following low-speed centrifugation of such a preparation (Figure 6-4), presumably because this method of lysis is harsher than homogenisation so that organellar membranes become more fragmented (see Chapter 5) and remain in the supernatant of a low-speed centrifugation.

Figure 6-5 shows that it is possible to recover all the fusion protein produced by JRY188(pNB66) from a glass bead lysate of such cells using IgG-Sepharose. If SpaK resided in the Kex2 compartment such a result would led to the prediction that all of the Kex2p of the cell would also be recovered. This was found not to be the case (Figure 6-5). Analysis of the bound fraction obtained from JRY188(pNB66) using IgG-Sepharose revealed that it contained over a third of the total amount of DPAP B (a vacuolar membrane protein: Bordallo *et al.*, 1984; Roberts *et al.*, 1989) found within

the lysate and also a significant amount of a luminal vacuolar enzyme, CPY (Figure 6-5). The fact that CPY is a soluble enzyme (Wiemken *et al.*,1979) may explain why a smaller proportion of the total amount of CPY is found in the fraction recovered using SpaK (smaller than the proportion of the membrane protein DPAP B found in the same fraction); it is likely that CPY escapes from the vacuole during lysis of the cells (the organelle will become broken up into vesicles and not all of the vacuolar contents will be incorporated into these).

The results presented above are consistent with the idea that the fusion protein is located to the vacuole. One finding that appears initially to be inconsistent with this conclusion is that PWYS3(pNB66) cells process pro-α-factor (as determined by a halo assay; data not shown) suggesting that the fusion protein is located in a compartment encountered by pro-α-factor as it travels through the secretory pathway. However, an assay which allows a more reasonable analysis of the levels of α-factor production was carried out on these cells (Figure 6-6) and it was found that although PWYS3(pNB66) cells do process the pheromone they do not do so to the same extent as cells that contain an intact *KEX2* gene. It may be that SpaK processes pro-α-factor as it travels through the Golgi on its way to the vacuole. This would explain why PWYS3(pNB66) cells secrete less processed pheromone than JRY188 cells since the levels of SpaK in the Golgi at any one time will be considerably lower than the levels of Kex2p in the same compartment of cells producing Kex2p.

Although it was not possible to use SpaK to immunoisolate the same material that was recovered using Kex2-ImAd, it has been demonstrated here that the attachment of protein A domains onto an integral membrane protein to construct a fusion protein allows the use of IgG-Sepharose to recover the membrane in which the fusion protein resides. The experiments described here demonstrate the importance of thorough characterisation of hybrid proteins. It was assumed here that SpaK would adopt the

same cellular localisation as Kex2p, but this does not appear to be the case. Further characterisation of SpaK, and the material isolated using its affinity for IgG, may provide a basis for the development of a procedure to allow immunoisolation of the yeast vacuole.

Figure 6-4.

Fractionation of SpaK

A 100ml culture of JRY188(pNB66) was divided in half. Cells from one half of this culture were lysed using glass beads and the remainder were lysed using homogenisation following removal of their cell wall by zymolyase. The preparations yielded by both of these procedures were subjected to centrifugation at 4000g for 2 mins giving rise to a pellet (P4) and a supernatant (S4).

Two aliquots of each of the various samples thus obtained (total extract, P4 and S4) were subjected to electrophoretic separation on a 6% SDS-polyacrylamide gel, and then were transferred to nitrocellulose. One portion of this filter was tested for reactivity with a rabbit IgG-HRP conjugate (which will recognise SpaK), shown as the top immunoblot. Another portion of the filter was tested for reactivity with anti-Kex2C which will recognise both SpaK and Kex2p (bottom).

method of cell lysis	glass bead lysis			homogenisation		
fraction	S4	P4	lysate	S4	P4	lysate

SpaK

SpaK

Kex2p

Figure 6-5.

Recovery of SpaK-containing membranes

50μl (slurry volume) aliquots of IgG-Sepharose were used to recover material from 300μl (equivalent to 30OD units) of an S4 prepared from a glass bead lysate of a culture of JRY188(pNB66) in the following way: The IgG-Sepharose was washed 6 times with lysis buffer before being resuspended in the S4. Following incubation on a rotating wheel at 4°C for 3 hours the IgG-Sepharose was recovered by centrifugation at 2000g for 2 mins. The IgG-Sepharose was washed 3 times with lysis buffer (this washed sample was taken as the bound material) and was then either boiled in SDS sample buffer prior to immunoblot analysis using either a rabbit IgG-HRP conjugate (Figure 6-5a) or anti-Kex2C (Figure 6-5b) following electrophoretic separation on a 10% SDS-polyacrylamide gel and transfer to nitrocellulose, or was assayed for the activities of various enzymes (Figure 6-5c).

The same procedure was carried out using extract prepared from JRY188 as a control and the material remaining in the extract following removal of the IgG-Sepharose was also analysed (unbound material). All of the analyses described here were also carried out on a sample of each of the glass bead extracts (lysate), and the enzyme activity assays are expressed as a percentage of that found in the lysate from which the bound fraction was obtained.

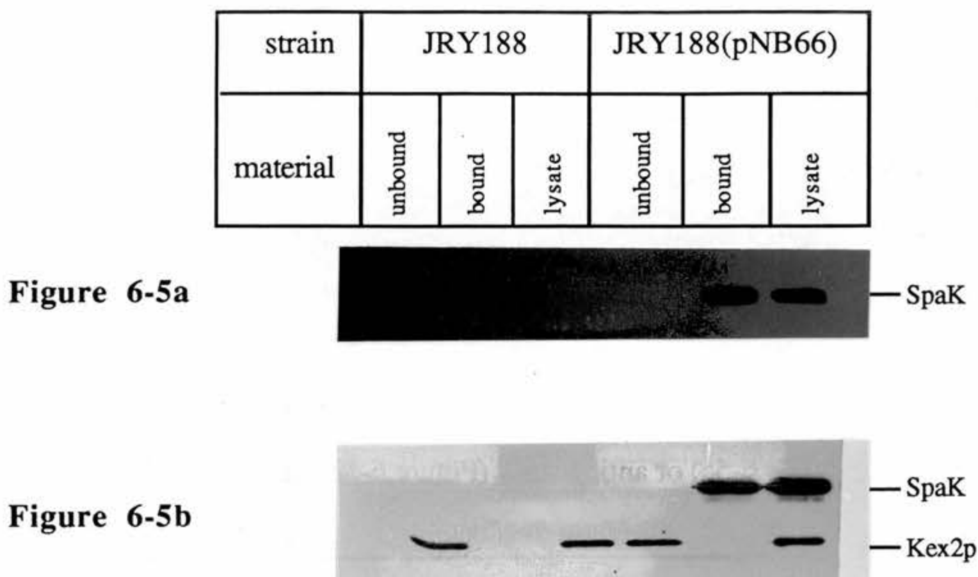
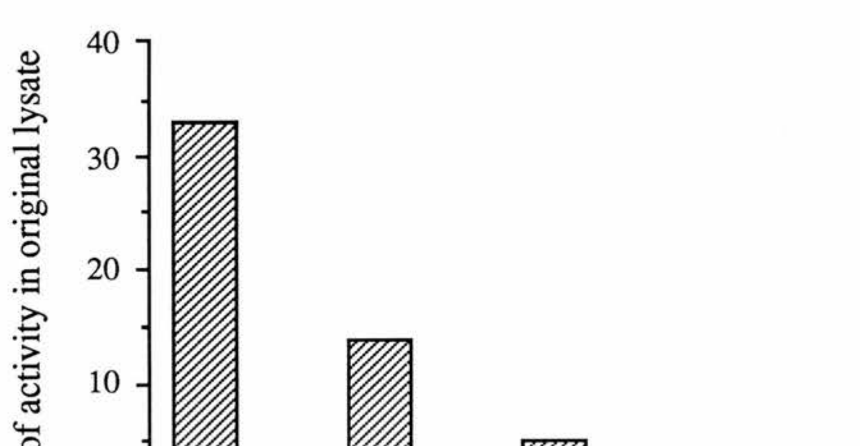


Figure 6-5c



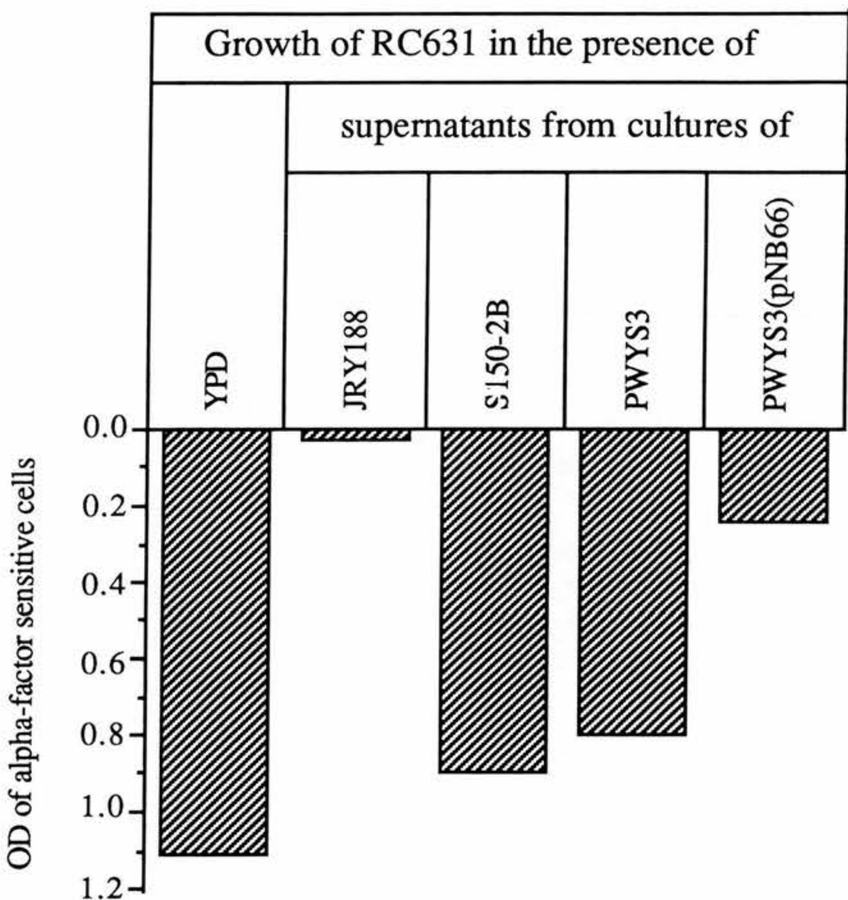
activity present in material recovered from JRY188(pNB66)

activity present in material recovered from JRY188

Figure 6-6.

Pro-α-factor processing by SpaK

The ability of the yeast strain RC631 (whose growth is sensitive to the presence of α-factor) to grow in the presence of supernatants from various yeast cultures (taken at OD_{600}=0.5) was tested in order to ascertain whether these cells were processing pro-α-factor. The growth of RC631 in the presence of sterile YPD was also measured.



Growth of RC631 in the presence of
supernatants from cultures of
YPD
JRY188
S150-2B
PWYS3
PWYS3(pNB66)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
OD of alpha-factor sensitive cells

Chapter 7

Discussion

Discussion

The product of the *Saccharomyces cerevisiae KEX2* gene is a Ca^{2+} dependent, neutral serine protease that cleaves peptide substrates at the carboxyl side of Lys-Arg and Arg-Arg sites. Kex2p is required for maturation of pro-α-factor, a process that occurs before fusion of secretory vesicles with the plasma membrane, in the Golgi apparatus. Kex2p can accurately process the mammalian precursors proinsulin and pro-opiomelanocortin *in vivo*, and its similarity to proteases that carry out these processing events in mammalian cells is one piece of evidence that has led to the conclusion that Kex2p resides in a compartment in yeast cells that is functionally equivalent to the *trans* Golgi network of mammalian cells (Wilcox and Fuller, 1991: others are discussed in Chapter 1).

During the course of this project I set out to develop a procedure to use Kex2p to isolate the cellular compartment in which it resides so that this organelle may be characterised. To this end, bacterially-produced hybrid proteins consisting of the, normally cytoplasmically-disposed, C-terminal 100 amino acids of Kex2p fused to either protein A or βgal, were used to raise, and affinity purify polyclonal antibodies that specifically recognise this region of the protein (anti-Kex2C: Chapter 3). These antibodies were attached to *S. aureus* cells, to form a Kex2-ImAd, which was subsequently used to recover material, based on the affinity of anti-Kex2C for Kex2p, from yeast cells (Chapter 4). Conditions were found that led to the recovery of Kex2p contained within intact membrane vesicles. As well as being highly enriched for the Kex2 protease, the immunoisolated material also contains the two pheromone processing enzymes which act after Kex2p in the maturation of pro-α-factor (Kex1p and DPAP A: Fuller *et al.*, 1988). From this work, it seems that all three of the enzymes involved in the maturation of pro-α-factor to α-factor reside in the same cellular compartment. This provides further evidence that the yeast Kex2p-containing compartment is analogous to the TGN of mammalian cells, since if Kex1p and DPAP A did not reside in the same compartment as Kex2p, then the mating pheromone would have to encounter at least one other cellular compartment after leaving the Kex2p-containing compartment prior to

secretion in order to be fully processed, but the finding that Kex2p, DPAP A and Kex1p all reside in the same compartment means that it is possible (and likely) that the pheromone does not encounter any other Golgi compartment after the Kex2p-compartment. The immunoisolated material does not contain any activity associated with enzymes that are associated with the ER (NADPH-cytochrome *c* oxidoreductase) or the vacuole (DPAP B or CPY). This apparent lack of contamination of the membrane fraction has been confirmed by immunoblot analysis using an antiserum which recognises a second ER membrane protein (HMG-CoA reductase), and it is envisaged that immunoblot analysis of the same fraction with an antibody that recognises the vacuolar enzyme CPY would detect the protein in its inactive precursor form *en route* to the vacuole. The ImAd recovered material has been shown to contain a plasma membrane protein (Pma1p), believed to be recovered in association with the Kex2p-compartment as traffic *en route* to its cellular location. From the results presented in Chapters 4 and 5, it seems reasonable to conclude that the material recovered by immunoisolation, based on the affinity of a polyclonal antibody for the cytoplasmically-disposed C-terminal domain of Kex2p, is representative of the yeast Kex2p-compartment, the yeast counterpart of the mammalian TGN.

An interesting finding from the experiments designed to optimise the recovery of Kex2p-containing membranes from yeast cells, was that the co-recovery of DPAP A with Kex2p was dependent on the method used to lyse the yeast cells, with such co-recovery only being achieved following gentle lysis of the cells. A model to explain this finding is suggested here; if the membrane of the Kex2-compartment is divided into domains, with Kex2 being found in one domain, and DPAP A in another. In such a model, when harsh lysis conditions disrupt the compartment, the resultant vesicles are unlikely to contain both of the enzymes (since they are spatially separated), whereas gentle cell lysis will cause less fragmentation of the compartment so that the liklihood of recovering the intact organelle is higher.

A second approach that was taken to isolate the Kex2p-compartment involved the construction of a gene fusion which, when transformed into yeast cells, caused them to produce a hybrid protein, consisting of IgG-binding domains of the protein A, fused to the extreme C-terminus of Kex2p (SpaK: Chapter 6). Material recovered from yeast cells using this protein's affinity for IgG does not contain normal Kex2p (also present in these cells JRY188(pNB66)), and is therefore believed not to be the Kex2p-containing compartment. Analysis of this material suggests that it is vacuolar in origin. This suggests that the fusion protein does not take up residence in the Kex2p-compartment as was expected, but is located in the vacuole. While this work was being carried out, it was reported that mutations in the C-terminal domain of Kex2p cause the protein to be transported to the vacuole (Roberts *et al.*, 1992: Seeger and Payne, 1992). This could be explained by speculating that these mutations cause changes to a protein's structure and it is these changes that cause the cell to transport the abnormal protein to the vacuole to be degraded (by an unknown mechanism). However, it has been suggested that transport to the vacuole is the default pathway for yeast membrane proteins, and changes in the C-terminus of Kex2p (and indeed in the cytoplasmic domain of other yeast Golgi membrane proteins) destroy a signal (as yet unidentified) that serves to retain the protein in the Golgi (Fuller *et al.*, 1989b). Further evidence for this hypothesis came from experiments designed to identify target signals that cause proteins to be transported to the vacuole. These involved the construction of various gene fusions and partial deletions using the genes that encode the two yeast integral membrane DPAPs; A (which resides in the Golgi) and B (which resides in the vacuole) (Roberts *et al.*, 1992). Removal or replacement of either the cytoplasmic, transmembrane, or lumenal domain of DPAP B does not affect its localisation to the vacuole, whereas the C-terminal domain of DPAP A is both necessary and sufficient to retain the protein in the Golgi. Overproduction of DPAP A results in some of the protein becoming localised to the vacuole, suggesting the saturation of a retention mechanism. One observation which seems to be inconsistent with the idea that the vacuole is the default pathway for yeast membrane proteins, is that *chc1* cells (which lack the heavy chain of clathrin) mislocalise Kex2p to the cell surface (Payne and

Schekman, 1989). This appears to suggest that the default pathway for the protease leads to its localisation here, but it may be that it is not a direct interaction of Kex2p with the clathrin heavy chain that prevents its transport to the vacuole. Kex2p could interact with another protein which, in turn, could interact with the clathrin heavy chain, and thus retain Kex2p in its rightful location. If this were the case, then in *chc1* mutants, the protein that interacts with the C-terminal domain of Kex2p would still serve to direct it away from the vacuole, but could not keep it in the Golgi due to the lack of clathrin heavy chain.

If the hypothesis described above is correct, and SpaK is localised in the vacuole, it is perhaps suprising that the protein is not degraded (a protein A fusion protein of the expected apparent molecular weight is produced by cells harbouring the gene fusion). It has been proposed that there is an organelle which is an intermediate between the Golgi complex and the vacuole (Seeger and Payne, 1992) which, if it exists, SpaK would encounter *en route* to the vacuole. This would be consistent with observations that have been made following the treatment of mammalian cells with the drug brefeldin A which suggest that some proteins encounter an endosomal compartment *en route* to the lysosome (Lippincott-Schwartz *et al.*, 1991). It may be that the material recovered throught SpaK's affinity for IgG is derived, not only from the vacuole, but also from this putative intermediate compartment. It is possible that the SpaK which reaches the vacuole its lumenal domain is degraded, leaving its trans-membrane and cytoplasmic domains remaining intact. The full length fusion protein detected in material recovered from cells expressing the gene fusion would, using this hypothesis, be resident to the intermediate compartment, and the vacuolar markers (e.g. the active, and therefore mature CPY) will have been recovered through the truncated SpaK in the vacuolar membrane (the polyacrylamide gels used in this work were of such a composition that would not allow the detection of such a small polypeptide; this may explain why no protein A fusion protein of a molecular weight consistent with this theory was detected here).

Such a scheme can also be used explain the observation that SpaK-producing *kex2* mutant cells process pro-α-factor to some extent, which seemed contradictory to the fusion protein's apparent localisation to the vacuole, but was explained in Chapter 6 as being due to SpaK in the Golgi *en route* to the vacuole processing the pheromone. If there is an organelle encountered by proteins after the Kex2p-compartment, before they reach the vacuole, which is also encountered by proteins *en route* to the plasma membrane, then pro-α-factor will encounter SpaK (which has Kex2 protease activity) in this organelle and thus become processed.

This work has led to the immunoisolation of a membrane fraction consisting of vesicles derived from the Kex2p-containing compartment of the yeast Golgi, and it is hoped that this material will be used to develop protein transport assays which reconstitute some of the *in vivo* functions of this organelle. Obviously, for such assays to be established, it is necessary that the immunoisolated Kex2p-containing compartment is 'functional', or 'competent' with regards to reconstituting protein trafficking events as they occur *in vivo* and preliminary experiments that reconstitute the delivery of a soluble cargo protein to the immunoisolated Kex2p-compartment (Chapter 5) go some way to demonstrating that this is the case. The detection of Pma1p in the isolated material may enable the process of budding from the Kex2p-compartment to be studied. This could be assayed for by following the appearance of this cargo protein in a supernatant that would result following the removal of immunoisolated Kex2p-containing compartment from the assay (by centrifugation). It would be important to distinguish between budding of membranes and fragmentation of the immunoisolated material, and this could be achieved by demonstrating that the appearance of Pma1p in the supernatant had an energy requirement. Such an assay could be used to identify proteins that are important in the budding of membrane vesicles from the Kex2p-containing compartment. For example, a number of *sec* mutants are thought to be defective in the budding of vesicles from the yeast ER (Kaiser and Schekman, 1990) and the proposed assay may be of use in determining whether the proteins involved in this process are also required for the budding of vesicles from the Kex2p-compartment.

This work demonstrates the power of the technique of immunoisolation, and has created a number of possible openings which may now be taken to study the function(s) of the hitherto poorly understood Kex2p-continaing compartment.

Appendix

A-1. Table 1. Bacterial and yeast strains used in this study

	Genotype	Source
Bacterial		
E. coli NM522	*Δ (lac-proAB) hisΔ5 (rk-, mk+) thi supE F/ (proAB lacIq) ZΔM15*	Gough and Murray (1983)
E. coli pop2316	*F- supE E44 hsd R17 mcrA+mcrB+ rk- mk+ endA1 thi-1 aroB mal-λ-*	Kusters *et al.* (1989)
Yeast *(Saccharomyces cerevisiae)*		
JRY188	*MATα leu 2-3, 112 ura3-52 trp1 his4 sir3-8 rme GAL*	Brake *et al.* (1984)
PWYS3	*MATα leu 2-3, 112 ura3-52 trp1 his4 sir3-8 rme GAL (kex2::URA3)*	P. Whitley (JRY188 derivative)
RC631	*MATa sst2 rme ade2 ura1 his6 met1 can1 cyh2 GAL*	Chan and Otte (1982)
NBY10	JRY188(**a**/α diploid)	This study
DC17	*MATα his1*	J. Hicks
DC14	*MATa his 1*	J. Hicks

A-2. Table 2. Plasmids used in this study

Table 2a. Plasmids obtained from others

pGA714	G. Ammerer	5kb yeast genomic *KEX2* fragment cloned in YEp13
pK19	R.D. Pridmore	Derivative of pUC19: Km^R replaces Ap^R (Pridmore, 1987)
YCplac22	D. Geitz	*CEN4/TRP1* plasmid (Geitz and Sigino, 1988)
pKpra	A. Boyd	*spa* fusion vector (Zueco and Boyd, in press)
pEX11, 12 & 13	J.G. Kusters	*lac* Z fusion vectors (Kusters *et al.*, 1989)
pAX11, 12 & 13	J. Zueco	*spa* fusion vectors (Zueco and Boyd, in press)
pAB510	M. Egerton	pBR322 carrying a 4.3kb *Bam*HI fragment containing *STE2* gene
pIH2-4	M. Egerton	*URA3*, YEp plasmid with *STE2* gene under control of the *GAL1* promoter

Table 2a. (continued)

pYJS50	A. Boyd	*LEU2*, YEp plasmid carrying an MFα1-*bla* gene fusion
pUC18	A. Boyd	(Messing, 1983)
pGAL-HO	I. Herskowitz	*CEN4-URA3* plasmid carrying *HO* gene
pKpraKex2C	P. Whitley	1.25kb *Eco*RI/*Bam*HI fragment from pGA714 cloned into similarly digested pKpra

Table 2b. Plasmids constructed in this study

pKpraSB	self-annealed oligonucleotide AGCTGGTCGACC cloned into the *Hin*dIII site of pKpra
pKpraSH	a linker formed from the two oligonucleotides AATTGTAAGCTTG & GATCCAAGCTTAC cloned into *Eco*RI/*Bam*HI digested pKpraSB
pNB1	1.6kb *Hin*dIII fragment, containing *STE2*, cloned into the *Hin*dIII site of pK19, orientated to give a 1.6kb fragment upon digestion with *Hin*dIII
pNB2	0.8kb *Hae*III fragment from pNB1 cloned into the *Sma*I site of pUC18, in such orientation that yields a 0.6kb *Hin*dIII fragment

pNB3	0.8kb *Kpn*I/*Bam*HI fragment from pNB2 cloned into similarly digested pKpra
pNB4	Cohesive ends created following *Sal*I digestion of pNB3 were made blunt using Klenow fragment, prior to re-ligation
pNB13	0.8kb *Hae*III fragment from pNB1 cloned into *Sma*I site of pEX11, in orientation that gives 500bp *Sal*I fragment
pNB21	1.25kb *Eco*RI/*Bam*HI fragment from pKpraKex2C cloned into similarly-digested pEX11
pNB64	3.6kb PCR product obtained using the two oligonucleotides GCGGATCCGTCGATCGTCCGGAAGATGG & CGGGATCCGGTACCTCCAGTGCAACCAAACG as primers and genomic DNA prepared from NBY10 as template cloned into *Bam*HI/*Kpn*I digested pK19
pNB65	0.5kb *Sal*I/*Hin*dIII fragment encoding protein A IgG binding domains cloned into *Sal*I/*Hin*dIII digested pNB64, placing this fragment downstream of *KEX2*
pNB66	3kb *Bam*HI fragment containing *KEX2-spa* gene fusion cloned into the *Bam*HI site of YCplac22

pNB72 1.6kb PCR product (encodes from Ile_{524} to Ser_{1054} of HMG-CoA reductase) obtained using the oligonucleotides GAATGCTGCTAGAATTCATACCAG & CGGGATCCAATGACGTATGACTAAGTTTAGG as primers and genomic DNA prepared from NBY10 as template DNA cloned into *Eco*RI/*Bam*HI digested pAX12

pNB73 1.6kb *Eco*RI/*Bam*HI fragment from pNB72 cloned into similarly digested pEX12

pNB75 0.54kb fragment (encoding from Ala_{354} to Asp_{534} of the yeast plasma membrane H^+ ATPase, Pma1p) obtained by PCR using the oligonucleotides CTCTTGGTGGATCCATACATGG & TGGCCCGGGCTAAGAAAACAAGCCATTGTTC as primers and genomic DNA prepared from NBY10 as template DNA cloned into *Sma*I/*Bam*HI digested pAX11

pNB76 0.54kb *Sma*I/*Bam*HI fragment from pNB75 cloned into similarly digested pEX11

A-3. Creation of the yeast strain NBY10

To create the strain NBY10, JRY188 was transformed with the pGAL-HO (a plasmid harbouring a copy of the *HO* gene under *GAL1* control carrying the *URA3* marker). The resultant transformants were grown on YPG plates to induce expression of the *HO* gene. Expression of this gene causes cells to change mating type from **a** to α and vice versa nearly every cell division (Herskowitz and Jensen, 1991). Any single colony on a YPG plate is therefore likely to, at any given time, contain haploid cells of both mating types and siblings of opposite mating types will be able to mate with each other to form **a**/α diploids.

A single colony, putatively containing **a**, α and **a**/α diploids, was streaked from YPG onto YPD in order to seperate the three possible cell types and switch off expression of the *HO* gene. Ten single colonies were then replica patched from this plate onto YPD and sporulation media plates. After a weeks incubation at 30°C, cells from the sporulation media plates were scored for asci. Although no asci could be seen the cells appeared larger and rounder than cells which had not been subjected to sporulation conditions and therefore further steps were taken to analyse the cells.

Figure A-1 shows that cells from all of the ten colonies picked do not secrete α-factor, this would be the expected result had an **a**/α diploid been picked. However this result would also be obtained if a haploid cell of the '**a**' mating type had been picked, such a stable '**a**' type cell could arise had a cell lost the plasmid harbouring the *HO* gene while on rich media.

It was important that the strain obtained had lost the plasmid harbouring the *HO* gene after **a**/α diploid formation, during its time on rich media. This is partly because the plasmid carries the *URA3* marker, as does pIH2-4 so in order for positive transformants from the transformation of NBY10 with pIH2-4 to be selected it was necessary for the strain to be Ura-. The ten patches from the YPD plate were patched onto minimal media that had been supplemented with /-

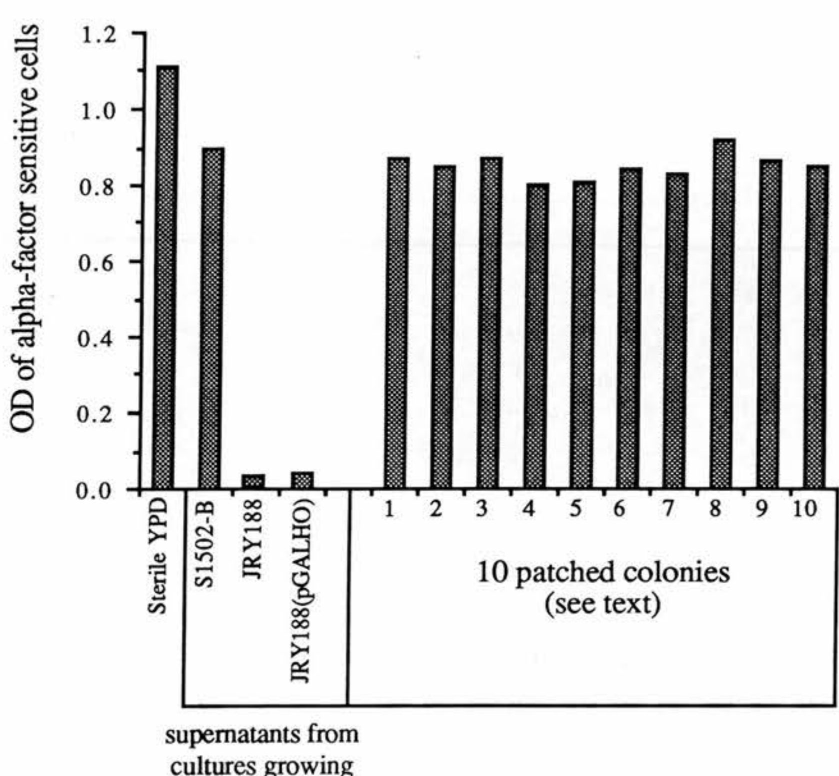
1. His, Ura, Trp and Leu.
2. His, Ura and Trp.
3. His, Ura and Leu.
4. His, Trp and Leu.
5. Ura, Trp and Leu.

All of the patches grew on all five plates with one exception, 'patch number 8' did not grow on the plate that had not been supplemented with uracil (i.e. plate number 4). These cells have lost the *HO* containing plasmid.

Cells from 'patch number 8', taken from YPD, were mixed seperately with DC14 (*MATa*) and DC17 (*MAT*α) cells on minimal media plates. Both of these strains were also mixed with JRY188 (*MAT*α) and S1502B (*MATa*) as positive controls (with DC14 mating with JRY188 and DC17 mating with S1502B). Neither DC14 nor DC17 mated with 'patch number 8' and this was taken as a final conformation that the **a**/α diploid had been formed, and the new strain was labelled as NBY10.

Figure A-1.

Screening for a/α diploid formation by loss of α-factor production. Ten JRY188(pGAL-HO) colonies that had been grown on galactose containing media (SD+his, trp and leu) to ionduced expression of the *HO* gene, and then moved to glucose containing media (YPD - as described in the text) were assayed for the production of α-factor using the strain RC631, whose growth is sensitive to the presence of the mating pheromone. Also assayed for secretion of the pheromone here are the strains JRY188 (α) and S1502-B (**a**), as well as the transformant JRY188(pGALHO) which has never expressed the *HO* gene harboured by pGAL-HO. The growth of RC631 in the presence of sterile YPD was also measured.



OD of alpha-factor sensitive cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sterile YPD
S1502-B
JRY188
JRY188(pGALHO)
1 2 3 4 5 6 7 8 9 10
10 patched colonies
(see text)
supernatants from
cultures growing
in YPD

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